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Supplementation with docosahexaenoic acid and 5-methyltetrahydrofolate during the second half of pregnancy – effects on placental fatty acid profile, apoptosis and proliferation

Dissertation

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Abbreviations

BHT	Di.-tert.-buthyl-p-cresol
BMI	Body mass index
CE	Cholesterolester
CV	Coefficient of variation
EFA	Essential fatty acids
FAME	Fatty acid methyl esters
FAPB	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transfer protein
GLC	Gas liquid chromatography
H-FABP	Heart fatty acid binding protein
HRP	Horseradish proteinase
LC-PUFA	Long-chain polyunsaturated fatty acids
L-FABP	Liver fatty acid binding protein
M30	Cytokeratin 18 neoepitope
MTHF	Methyltetrahydrofolate
MUFA	Monounsaturated fatty acids
NEFA	Nonesterified fatty acids
PCNA	Proliferating cell nuclear antigen
PL	Phospholipide
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
TG	Triglyceride
TLC	Thin layer chromatography

Fatty acids:

AA	Arachidonic acid (C20:4n-6)
ALA	α-linolenic acid (C18:3n-3)
DGLA	Dihomo-γ-linolenic acid (C20:3n-6)
DHA	Docosahexaenoic acid (C22:6n-3)
DPA	Docosapentaenoic acid (C22:5n-3/n-6)
EPA	Eicosapentaenoic acid (C20:5n-3)
LA	Linolic acid (C18:2n-6)

1 Introduction

1.1 Requirements during pregnancy

During pregnancy nutritional and energy requirements increase, primarily due to maternal and foetal tissue accretion (1). In Europe pregnant women can easily meet their enhanced energy and protein needs (2), with the exception of special subgroups such as women with eating disorders or gastrointestinal diseases. However, the pregnancy associated increase of the requirements of some micronutrients and n-3 fatty acids is far higher than the enhancement in energy requirement (1). The recommended increase of energy intake during pregnancy is 17-22%, whereas the reference intakes for protein, and some vitamins and minerals, as folate, iodine, vitamin B-6 and iron, increase by approximately 50% and for DHA by approximately 40% (Table 1.1) (1;3).

Table 1.1: Comparison of recommended daily energy and nutrient intakes of adult and pregnant women (1;3).

Energy / Nutrient	Adult	Pregnancy	Nutrient	Adult	Pregnancy
Energy[1]	19-50 y	+ 340 kcal/d	Pantothenate (mg)[2]	5	6
		2nd trimester	Biotin (µg)[2]	30	30
		+ 450 kcal/d	Vitamin A (µg)[1]	30	30
		3nd trimester	Vitamin D (µg)[2]	5	5
			Vitamin E (mg)[1]	15	15
Protein (g)[1]	46	71	Vitamin K (µg)[2]	90	90
DHA (mg)[2]	220	300	Calcium (mg)[2]	1000	1000
Vitamin C (mg)[1]	75	85	Phosphorus (mg)[2]	700	700
Thiamin (mg)[1]	1.1	1.4	Magnesium (mg)[1]	310	350
Riboflavin (mg)[1]	1.1	1.4	Iron (mg)[1]	18	27
Niacin (ng)[1]	14	18	Zinc (mg)[1]	8	11
Vitamin B_6 (mg)[1]	1.3	1.9	Iodine (µg)[1]	150	220
Folate (µg)[1]	40	600	Selenium (µg)[1]	55	60
Vitamin B_{12} (µg)[1]	2.4	2.6	Fluoride (mg)[2]	3	3

[1]Recommend Dietary Allowance (RDA), average daily dietary intake level that is sufficient to meet the nutrient requirements of almost all (97-98%) individuals in a life stage and gender group based on Estimated Average Requirements (EAR) (3).

[2]Adequate Intake (AI), the value used instead of RDA, if adequate scientific evidence is not available to calculate EAR (1).

1.2 Docosahexaenoic acid during late uterine and early postnatal life

The n-3 fatty acid docosahexaenoic acid (DHA) is the predominant long-chain polyunsaturated fatty acid (LC-PUFA) in the central nervous system. It accumulates rapidly in brain and retina during late intrauterine and early postnatal growth (4-6). Available estimates suggest that approximately 67 mg n-3 fatty acids per day are accumulated in foetal tissue during the last semester of gestation (7). Estimates for the amounts of n-3 fatty acids accumulated in placental and maternal tissue are not available. Reduced brain and retinal DHA results in declined visual function and altered learning, behaviour, and neurotransmitter metabolism (8-10). It was reported that a dietary source of DHA increases n-3 LC-PUFA concentrations in foetal and infant plasma (11;12), which is associated with the improvement of an early development of visual acuity and other indexes of neurodevelopment in premature infants (13;14). In contrast to essential fatty acids (EFA), LC-PUFA percentages in the lipids of cord plasma are higher than in maternal plasma at the time of birth (15;16). Since the ability of the foetus and the human placenta to desaturate and elongate EFA, i.e. linolenic acid (LA, 18:2n-6) and α-linolenic acid (ALA, C18:3n-3), is limited (17), a selective materno-foetal transfer of DHA and arachidonic acid (AA) has been suggested. Despite this, the maternal dietary intake and plasma concentrations of DHA directly influence the DHA status of the developing foetus (18-20). Furthermore, recent studies have reported more mature electroencephalography patterns in newborn infants with higher plasma phospholipid DHA (19).

As structural components of cell membranes, LC-PUFA, as DHA and AA, influence membrane permeability, receptor functions and membrane associated enzyme activities (21). DHA and AA can be formed in the liver from the dietary EFA, α-linolenic acid (ALA, C18:3n-3) and linoleic acid (LA, 18:2n-6) (22) (23)(Figure 1.1). Although LA and ALA are formed in plants, they cannot be formed in mammalian cells because of the absence of the Δ12 and Δ15 enzymes necessary to insert a double bond at the n-6 or n-3 position of a fatty acid carbon chain. Stable isotope tracer studies suggest that less than 1-4% of dietary ALA is converted to DHA (24;25), raising the question of the possible importance of dietary DHA in humans (26). In this regard, dietary DHA results in higher concentrations of DHA in tissue phospholipids (PL) and higher DHA accretion than does its ALA precursor (27). Higher intakes of ALA fail to enhance plasma DHA in infants and adults (28).

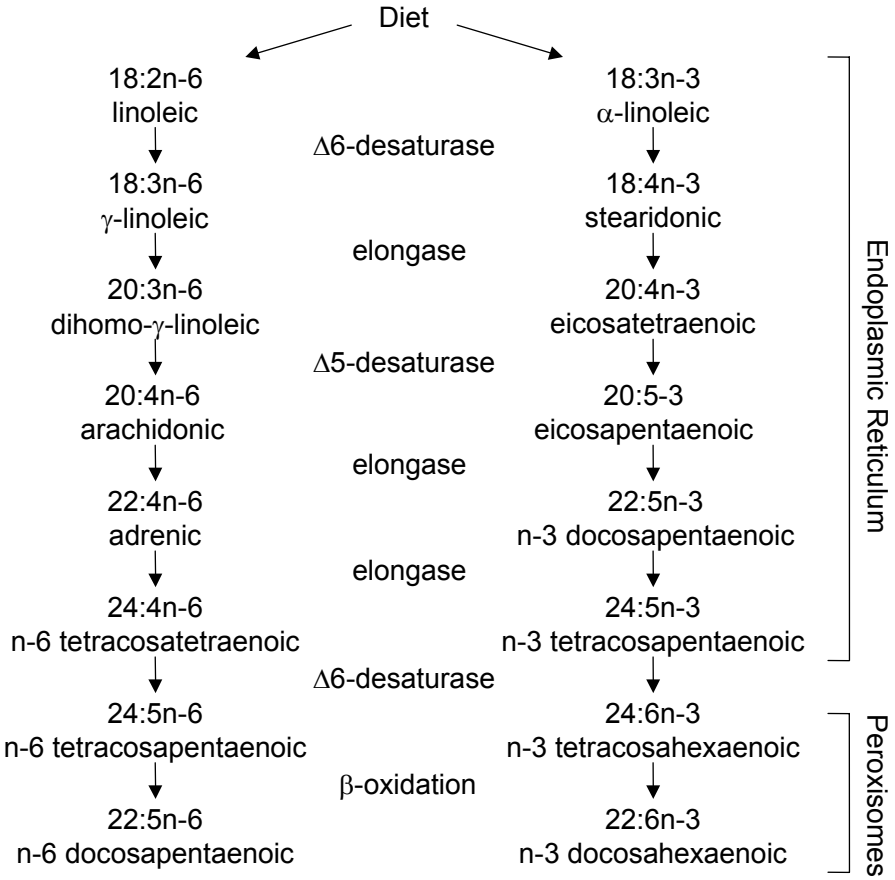


Figure 1.1: Biosynthesis of long-chain polyunsaturated fatty acids

1.3 Fatty acid transfer across the placenta

Fatty acids for placental transfer derive from a mixture of triglycerides (TG) and non-esterified fatty acids (NEFA) from maternal circulation. Only NEFA can enter the trophoblast, but the presence of lipoprotein lipase on the maternal surface enables the utilisation of TG (29;30). The placental uptake of fatty acids is a complex process involving several cytosolic and membrane associated fatty acid binding proteins (FABP) and transfer proteins (31). Observational studies indicate a preferential transfer of LC-PUFA to the foetal circulation relative to EFA (15;32). This is in agreement with in vitro studies, which have described a higher affinity and binding capacity of individual FABP for AA and DHA compared with LA and ALA (33). Furthermore, in experiments using placenta perfusion techniques, different transfer rates for individual fatty acids are described (34). The selectivity for PUFA is DHA>ALA>LA>AA, if the placenta is perfused with fatty acids mixtures reflecting the maternal TG composition in the last trimester of pregnancy, whilst the preference changes to

DHA>AA>ALA>LA, if the maternal perfusate contains fatty acids in the same ratios as found in circulating NEFA (35).

Once in the placenta, NEFA bound to FABP or transfer proteins, cross placental tissue in either direction, or are incorporated into PL, TG and cholesterolesters (CE) (36) (Figure 1.2).

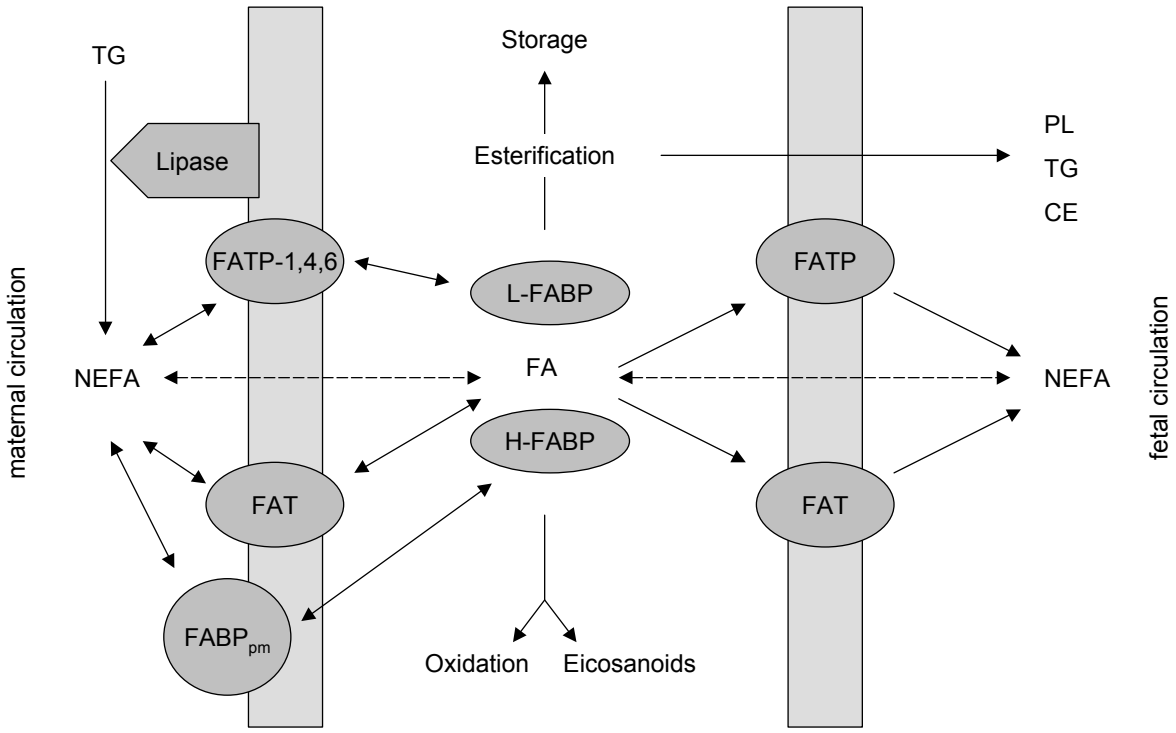


Figure 1.2: Distribution of fatty acid binding proteins in placental membrane (plasma membrane fatty acid binding protein; $FAPB_{pm}$, fatty acid translocase; FAT, and fatty acid transfer protein; FATP) and within the cytoplasm (Liver and Heart fatty acid binding protein; L- and H-FABP) of the trophoblast (31;37). Only fatty acids (FA) are released from maternal TG by lipoprotein lipase on the maternal surface of the placenta and the liberated FA, as well as NEFA from the circulation can be utilised by the placenta (38). The export of PL, CE and TG into the foetal circulation was reported (39). Adapted from (38).

The fatty acid composition of the NEFA pool is determined by NEFA derived from the maternal circulation. On the other hand, placental phospholipase A_2 or triacylglycerol hydrolase activity suggests that fatty acids could also be liberated from PL and TG (31;40), with subsequent accumulation in the NEFA pool. It was reported that DHA is incorporated in higher rates in TG than in PL (41), whereas AA is preferred esterificated in PL than in TG (39;41). Therefore, it is assumed that not only the NEFA fraction plays an important role in the foetal supply with n-3 and n-6 LC-PUFA, but also the PL and TG fraction. Data about the fatty acid compositions of placental NEFA, TG and CE fractions, which may be very important for the further understanding of

placental functions and transfer mechanisms, are not available and although the placental tissue is well-investigated, the underlying processes for placental fatty acid transfer, metabolism and accumulation have not been elucidated yet.

1.4 Effect of additional n-3 LC-PUFA intakes during pregnancy

The maternal plasma concentration of DHA directly influences the foetal DHA status (16), and the maternal fatty acid intake correlates with the n-3 LC-PUFA levels in their neonates (12). An additional daily intake of 1.2 g DHA or 2.7 g total n-3 LC-PUFA during pregnancy enhances concentrations of DHA in umbilical cord blood PL by 23% or 37%, respectively, compared with control groups (20;42). In contrast, the daily intake of 40 mg DHA from week 15 of pregnancy until birth was reported to increase only maternal plasma PL DHA levels by 27%, but not those of their new-born infants (43). Several authors reported beneficial effects of a high intake of seafood or DHA supplementation during gestation. In women with zero or low fish consumption, small amounts of n-3 LC-PUFA, provided as fish or fish oil, may improve the protection against preterm delivery and low birth weight (44).(45) Smuts et al. (2003) observed prolongation of pregnancy when DHA intake was increased during the last trimester (45). Such effects are not observed with the habitual intake of 25 mg DHA per day (46). A Norwegian study reported that a daily supply of 10 ml cod liver oil during pregnancy and lactation, improved Kaufman Assessment Battery for Children at four years of age by four percentages points (47). In these studies, adverse effects of the n-3 LC-PUFA supplementation on foetal growth and development have not been described (44;45;48).

1.5 Supplementation of 5-MTHF during pregnancy

Periconceptional folic acid supplementation decreases the occurrence of neural tube defects (49) and an additional folate intake during pregnancy may reduce pregnancy complications and adverse neonatal outcomes (50). Folate can enhance the conversion of homocysteine to methionine, yielding a reduction of plasma concentration of homocysteine (Figure 1.3).

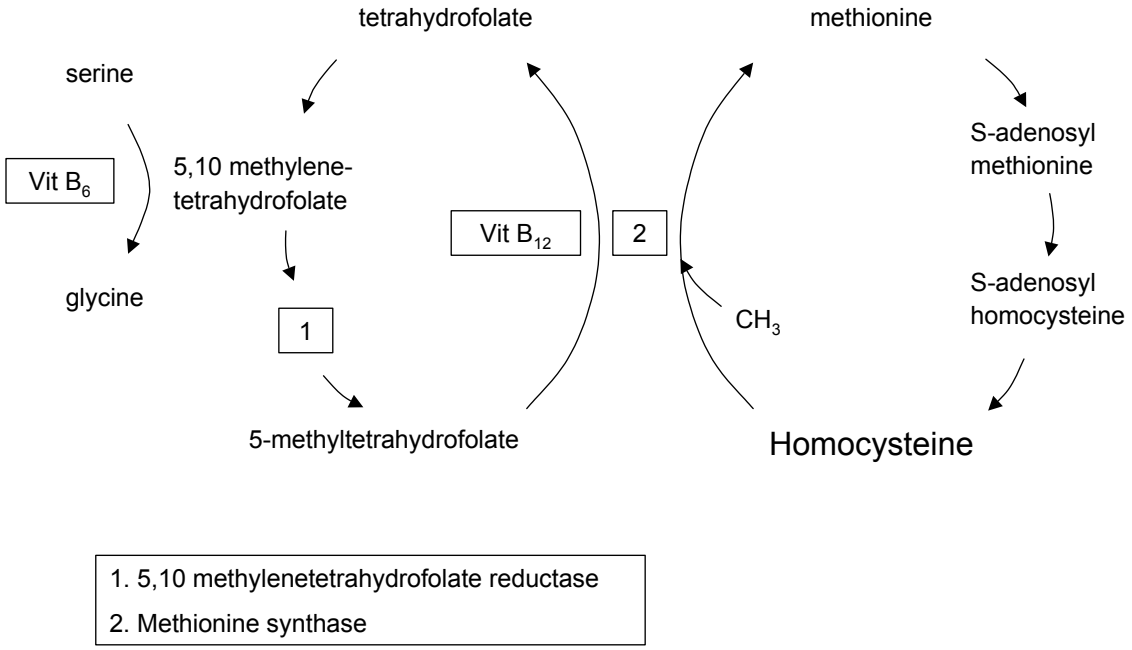


Figure 1.3: Remethylation of Homocysteine (51)

Moderately elevated plasma homocysteine concentrations are linked to propagation of vascular damage and increased rates of coronary heart disease (52). During pregnancy, reduction of elevated plasma concentrations of homocysteine might improve placental vascularisation, placental circulation and hence efficacy of materno-foetal substrate transfer. Indeed, a negative correlation between maternal plasma homocysteine and DHA-percentage in erythrocyte membrane PL of their new-borns was found, suggesting a possible positive influence of folate on DHA concentrations in cord blood of full term new-born infants (53). Thus, it appears possible that maternal folate supplementation during pregnancy additionally improves the DHA status of the neonate.

1.6 Homeostasis of apoptosis and proliferation in placental tissue

The placenta is a temporary organ that undergoes growth and development followed by senescence and death within nine month. The process of apoptosis (programmed cell death) was first described by Kerr et al. (1971) (54). Apoptosis is an active, regulatory response of inducible cells to specific stimuli that occurs only in cells having relevant response pathways (55). Several features of apoptosis distinguish the process from necrosis (56).

Proper placental function is of major importance for nutritional supply of the foetus. The morphological architecture and function of the human placenta depends on an adequate balance of proliferation, differentiation and apoptosis. In early pregnancy placenta cell proliferation, especially of cytotrophoblasts, is very high and then diminishes constantly with duration of pregnancy (57). In contrast, the apoptosis rate is low throughout early pregnancy and only increases shortly before delivery (58).
Recently, a number of studies have suggested that apoptosis plays a role in the normal development, remodelling, and ageing of the placenta (59-61). Villous cytotrophoblasts proliferate, differentiate, and merge into the syncytial layer by fusion. Aged syncytioplasm and nuclei are focally isolated within syncytial buds and are shed into the maternal circulation as syncytial sprouts (62) (Figure 1.4).

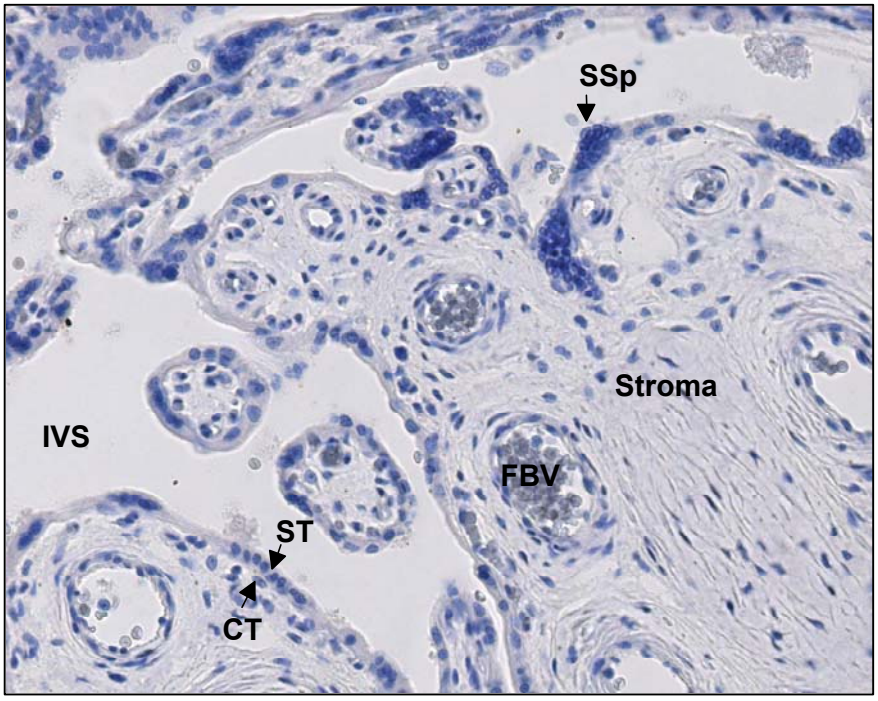


Figure 1.3: Full term placenta stained with Mayer's haemalumn (original magnification x 400).CT: cytotrophoblast; ST: syncytiotrophoblast; IVS: intervillous space; SSp: syncytium sprout; FBV: foetal blood vessel

Most of these aged nuclei are in some stage of apoptosis (60). Trophoblast turnover in villous tissue changes throughout normal pregnancy and some observations suggests that the rate of trophoblast apoptosis may change under certain pathologic conditions, such as spontaneous abortion, preeclampsia, preterm delivery or intrauterine growth retardation (63-65).

1.7 Objectives of this work

This work is intended to contribute a better understanding of the placental role by the supplementation of pregnant women with n-3 LC-PUFA. In a multicentre prospective

cohort study in Germany, Hungary, and Spain, pregnant women are consuming different diets supplemented from week 20 of pregnancy until child birth with either n-3 LC-PUFA, 5-MTHF, both or a placebo. The following questions define the objectives of this work:

1. The evaluation of a method for the analysis of fatty acid concentrations in placental tissue.
2. Comparison of the placental PL fatty acid pattern in the placebo supplemented group between the centres in relation to their different dietary backgrounds.
3. Effect of the additional intake with n-3 LC-PUFA and/or 5-MTHF on individual fatty acids in placental PL.
4. Influence of n-3 LC-PUFA and/or 5-MTHF on apoptosis and proliferation in placenta

2 Materials and Methods

2.1 Study design

2.1.1 Inclusion criteria

Eligible for the study were pregnant women, who fulfil the indicated inclusion criteria and were not going to participate in another clinical trial. Inclusion criteria were:

- Apparently healthy women with an uncomplicated singleton pregnancy
- Age ≥18 and ≤40 years at study entry
- weight >50 kg and <90 kg at study entry
- No use of fish oil supplements since the beginning of pregnancy
- No regular use of folate and/or vitamin B_{12} supplements after week 16 of gestation
- Intention to deliver in one of the obstetrical study centres

2.1.2 Application of the study diet

From November 2001 until September 2003 pregnant women were recruited between week 12 and week 20 of gestation in the University Hospitals of Granada, Spain, in the University of Pécs, Hungary, and in the Ludwig-Maximilians-University; Munich, Germany. All women habitually consumed an omnivorous diet. The participants were randomly and double blind assigned to one of four parallel study groups:

- n-3 LC-PUFA 500 mg DHA
- 5-MTHF 400 µg 5-MTHF
- Combined 500 mg DHA + 400 µg 5-MTHF
- Placebo -

The supplement was applied daily from week 20 of pregnancy.

Trough a lipase catalysed reesterification technique n-3 LC-PUFA was highly concentrated in triglycerides. The supplements contained an oil highly enriched with n-3 LC-PUFA (>60% of fatty acids, Pronova Biocare, Lysaker, Norway). The fatty acid

composition of the placebo and the n-3 LC-PUFA supplements are shown in Table 2.1.

Table 2.1: Fatty acid composition of the applied dietary supplements according to the manufactures analyses (% w/w). n.d.: not detected

	control	DHA / Combined
Fatty acid	mean (SD)	mean (SD)
C4:0	0.92 (0.02)	0.83 (0.07)
C6:0	1.41 (0.01)	0.92 (0.07)
C8:0	1.16 (0.01)	0.64 (0.02)
C10:0	3.07 (0.02)	1.57 (0.03)
C12:0	3.94 (0.03)	1.98 (0.01)
C14:0	12.88 (0.04)	7.33 (0.04)
C15:0	1.31 (0.00)	0.85 (0.00)
C16:0	36.55 (0.08)	22.05 (0.16)
C17:0	0.85 (0.00)	0.88 (0.01)
C18:0	10.37 (0.04)	8.82 (0.06)
C20:0	0.21 (0.00)	0.36 (0.01)
C16:1 n-7	2.13 (0.00)	1.63 (0.01)
C18:1n-9 + n-7	22.48 (0.05)	20.76 (0.13)
C20:1 n-9	n.d	1.01 (0.02)
C22:1 n-9	n.d	0.17 (0.01)
C18:2 n-6	1.74 (0.04)	2.02 (0.01)
C20:4 n-6	0.13 (0.00)	1.16 (0.01)
C22:4 n-6	n.d	1.01 (0.01)
C18:3 n-3	0.52 (0.02)	0.69 (0.02)
C20:5 n-3	n.d	5.58 (0.04)
C22:5 n-3	0.11 (0.00)	1.66 (0.02)
C22:6 n-3	n.d	17.84 (0.13)

The most active and abundant form of folate in tissues is 5-methyl-tetrahydro-folate. A new innovative technique enabled the enrichment in foods (BASF, Ludwigshafen, Germany). The components were supplied as 15 g milk based portions (Blemil plus, Ordesa Laboratorios, Barcelona, Spain), which contained vitamins and minerals in amounts meeting the estimated additional requirements during the second half of pregnancy (2) (Table 2.2).

Table 2.2: Nutrition, mineral and vitamin contents of the supplements

Nutrient content per sachet	*Combined*	*DHA*	*5-MTHF*	*Control*
DHA (mg)	500	500	-	-
5-MTHF (µg)	400	-	400	-
Energy (kcal)	71	71	70	70
Protein (g)	2.5	2.5	2.9	2.9
Fat (g)	3.1	3.1	2.9	2.9
Carbohydrates (g)	8.2	8.2	8	8
Vitamin A (µg)	330	330	330	330
Vitamin D (µg)	1.5	1.5	1.5	1.5
Vitamin E (mg)	3	3	3	3
Thiamin (mg)	0.36	0.36	0.36	0.36
Riboflavin (mg)	1.5	1.5	1.5	1.5
Niacin (mg)	4.5	4.5	4.5	4.5
Vitamin B6 (mg)	1.9	1.9	1.9	1.9
Vitamin B12 (µg)	3.5	3.5	3.5	3.5
Vitamin C (mg)	270	270	270	270
Calcium (mg)	300	300	300	300
Phosphorus (mg)	240	240	240	240
Magnesium (mg)	93	93	93	93
Zinc (mg)	3	3	3	3
Iodine (µg)	66	66	66	66

2.1.3 Investigated parameters in placental tissue

- Fatty acid profile (fatty acids with a chain-length of 14-24 carbon atoms) of PL, NEFA, TG and CE
- Total protein content
- Expression of proliferation cell nuclear antigen (PCNA) and p53
- Cleavage of cytokeratin 18 neoepitope

2.1.4 Parameters from the subject files

- Length of gestation (d)
- Placental weight (g)
- Infants' birth weight (g)
- Infants' birth length (cm)
- Maternal age (y)
- Body mass index at study entry (kg/m^2)
- Body mass index at delivery (kg/m^2)
- Maternal and foetal plasma folate (ng/ml)

The study protocol was approved by the local Ethical Committees of the participating centres. After a careful explanation of the study details, written consent was obtained from all participating women.

2.2 Materials and Equipment

2.2.1 Fatty acid analysis

2.2.1.1 Chemicals

Table 2.3: Chemical list for fatty acid analysis

Chemicals	Source	Quality
2.6 Di-tert.-buthyl-p-cresol (BHT)	Fluka, Neu-Ulm	99% GC
2.7 Dichlorofluorescein	Merck, Darmstadt	GR for analysis
2-Propanol	Merck, Darmstadt	LiChrosolv gradient grade
Acetic acid	Merck, Darmstadt	GR for analysis
Chloroform	Merck, Darmstadt	extra pure
Diisopropyl ether	Merck, Darmstadt	GR for analysis
Ethanol	Merck, Darmstadt	absolut p.a.
Methanol	Merck, Darmstadt	GR for analysis
Methanolic HCL (3N)	Supelco, PA, USA	p.a.
n-Heptane	Merck, Darmstadt	GR for analysis
n-Hexane	Merck, Darmstadt	LiChrosolv gradient grade
Potassium chloride	Merck, Darmstadt	GR for analysis
Sodium carbonate	Merck, Darmstadt	anhydrous GR for analysis
Sodium hydrogene carbonate	Merck, Darmstadt	GR for analysis
Sodium sulfate	Merck, Darmstadt	anhydrous GR for analysis
Water	Braun, Melsungen	ad injectabilia

2.2.1.2 Consumables supplies

Table 2.4: List for consumables supplies for fatty acid analysis

Materials	Source
Bottle G4, braun	CS-Chromatographie, Langerwehe
Bottle R1, braun/ 6.2 mm-BF	CS-Chromatographie, Langerwehe
Crimp cap R11-1.0	CS-Chromatographie, Langerwehe
Fiber glass filter, GMF 1, diameter: 100 mm	Sartorius, Bärenstein
Micro inlay G30/6	CS-Chromatographie, Langerwehe
Pasteur pipette	Brand, Wertheim
Pipette tip	Greiner, Frickenhausen
Screw cap G 13	CS-Chromatographie, Langerwehe
Sealing disc G13	CS-Chromatographie, Langerwehe
TLC plates, silica gel 60	Merck, Darmstadt
Tube for vacuum pump	Stricker, Oberschleißheim

2.2.1.3 Equipment

Table 2.5: Equipment list for fatty acid analysis

Equipment	Source
Analytical balance, R-200 D	Sartorius, Göttingen
Capper	Hewlett Packard, Böblingen
Centrifuge tube	Schmitz, Munich
Centrifuge, Universal 30 F	Hettrich, Tuttlingen
Di-Capper	Hewlett Packard, Böblingen
Megafuge, 1.0-R	Heraeus, Munich
Membrane vakuum pump, MZ 2c	Vacuubrand, Wertheim
Metal-block thermostat, Typ 2102	Bachhofer, Reutlingen
Metal-blade homogeniser, DIAX 100 + 8G/100	Heidolph Schwabach
Pipette 10-100µl, 50-250µl, 200-1000µl, 500-2500 µl	Eppendorf, Wesseling-Berzdorf
Rotavapor, R-114	Büchi, Flawil, Switzerland
Solvent chamber for thin layer chromatography	Desega, Heidelberg
Thermo plate, S	Desega, Heidelberg
Ultrasonic bath, Sonorex Super	Badelin, Berlin
Ultraviolet lamp	Benda, Wiesloch
Vortexter, VF 2	IKA, Heitersheim
Waterbath, B-480	Büchi, Flawil, Switzerland
Gas chromatograph	
Autosampler, 7673	Hewlett Packard, Böblingen
Capillary column, BPX-70, 60m, diameter 0.32 mm, film thickness 0.25	SGE, Weiterstadt
Gas chromatograph, 5890, Series 2	Hewlett Packard, Böblingen

2.2.1.4 Standards

Table 2.6: List for fatty acid analysis standards

Standard	Source
Internal standard:	
Phosphatidylcholine Dipentadecanoyl	Sigma, Deisenhofen
Pentadecaonic acid	Sigma, Deisenhofen
Tripentadecanoin	Sigma, Deisenhofen
Cholesteryl Pentadecanoate	Sigma, Deisenhofen
Standard for fatty acid identification:	
Adrenic acid	Sigma, Deisenhofen
Docosapentaenoic acid	Omega Tech, CO, USA
GLC-85 (reference standards)	NuChek, MN, USA
Lignoceric acid	Sigma, Deisenhofen
n-3 eicosapentaenoic acid	Sigma, Deisenhofen
stearidonic acid	Sigma, Deisenhofen
trans-docosenoic acid	Sigma, Deisenhofen
trans-hexadecenoic acid	Sigma, Deisenhofen
trans-tetradecenoic acid	Sigma, Deisenhofen
trans-trans-octadecadienoic acid	Sigma, Deisenhofen

2.2.2 Western blot and Immunohistochemistry

2.2.2.1 Chemicals

Table 2.7: List of chemicals for Western blot and immunochemistry

Chemicals	Source
AEC substrate system	Lab Vision, Vienne, Austria
Antibody diluent	DAKO, Glostrup, Denmark
Biotinylated Goat Anti-Polyvalent	Lab Vision, Vienne, Austria
Bovine serum albumin	Sigma, Deisenhofen
Copper sulfate	Merck, Darmstadt
Developer for photography	Ilford, Chesire, England
Di-sodiumhydrogenephosphate-decahydrate	Merck, Darmstadt
DMEM culture medium	Invitrogen, CA, USA
Fetal calf serum	Hyclone, Utah, USA
Fixer for photography	Ilford, Chesire, England
Fungizone	Sigma, Deisenhofen
Kaiser glycerolgelatin	Merck, Darmstadt
Medium 199	Invitrogen, CA, USA
NuPAGE antioxidant	Invitrogen, CA, USA
NuPAGE LDS SDS sample buffer	Invitrogen, CA, USA
NuPAGE MES SDS running buffer	Invitrogen, CA, USA
NuPAGE reducing agent	Invitrogen, CA, USA
NuPAGE transfer buffer	Invitrogen, CA, USA
Penicilin/Streptomycin	Sigma, Deisenhofen
Phenolic reagent	Merck, Darmstadt
Potassium sodium tartrate	Merck, Darmstadt
Potassium hydrogenephosphate	Merck, Darmstadt
Proteatic Inhibitor Cocktail	Roche, Mannheim
Sodium carbonate	Merck, Darmstadt
Sodium chloride	Sigma, Deisenhofen
Sodium dodecyl sulfate	Sigma, Deisenhofen
Sodium orthovanadate	Sigma, Deisenhofen
Streptavidin peroxidase	Lab Vision, Vienne, Austria
Tris (hydroxymethyl)-aminomethan	Merck, Darmstadt
Ultra V block	Lab Vision, Vienne, Austria
Water, Milli-Q	Millipore, Schalbach
<u>Test kit for Immunodetection:</u>	
Western Breeze	Invitrogen, CA, USA

2.2.2.2 Consumables supplies

Table 2.8: List of consumables supplies for Western blot and immunohistochemistry

Material	Source
Hyper film ECL	Amersham, Buckinghamshire, England
Nitro-cellulose membranes	Invitrogen, CA, USA
NuPAGE 4-12% bis-tris gel	Invitrogen, CA, USA
Tissue tek	Sakura, Zoeterwoude, Netherlands
Pipette tip	Eppendorf, Wesseling-Berzdorf
Safe look tubes (2ml)	Eppendorf, Wesseling-Berzdorf

2.2.2.3 Equipment

Table 2.9: List of equipment for Western blot and immunohistochemistry

Equipment	Source
Analytical balacance, Model 770	Kern, Balingen
Biofuge, Model 15	Heraeus, Wien, Austria
Centrifuge, Avanti 30	Beckmann, Krefeld
Centrifuge, CS-6R	Beckmann, Krefeld
Certomat TC 2	Braun, Melsungen
Decloaking chamber	Biocare Medical, Vienna, Austria
Digitalcamera	Olympus, Hamburg
Electrophoresis cell + Equipment	Invitrogen, CA, USA
Embedding apparatus, Citadel 1000	DAKO, Golstrup, Denmark
Gel dryer, Model 543	Biorad, Munich
Hypercassette TM	Amersham, Buckinghamshire, England
Metal blate homogeniser, T18, basic	IKA, Heitersheim
Microscpoe, BX 41	Olympus, Hamburg
Microtom, HM 440 E	DAKO, Golstrup, Denmark
Microwave, MWD 1820 DUO	Bauknecht, Vienna, Austria
Milli-Q, Biocel	Millipore, Eschborn
Minishaker, MS-1	IKA, Heitersheim
Powersupply, Powerpac 1000	Biorad, Munich
Spectrometer, DU 640	Beckmann, Krefeld
Thermomixer	Eppendorf, Wessling-Berzdorf

2.2.2.4 Antibodies and Marker

Table 2.10: List of antibodies and marker for Western blot and immunochemistry

	Source
Antibodies (Monoclonal)	
PCNA (PC 10)	Santa Cruz Biotech., CA, USA
Cytoceratin 18 neoepitope	Roche, Mannheim
p53 (D0-1)	Santa Cruz Biotech., CA, USA
Marker	
Magic Mark	Invitrogen, CA, USA

2.2.3 Computer programs

Table 2.11: List of the applied PC programs

Programs	Manufacturer
AlphaEaseFC, Version 3.2.3	Alpha Innotech, CA, USA
AxioVision 3.0	Carl Zeiss, Oberkochen
Corel paint 8.0	Microsoft GmbH, Unterschleißheim
Excel 97, SR-2	Microsoft GmbH, Unterschleißheim
EZChrom Elite Client, Version 2.61	Scientific Software, CA, USA
Power Point 97	Microsoft GmbH, Unterschleißheim
SPSS, Version 11.5.1	SPSS GmbH, Software
Word 97, SR-2	Microsoft GmbH, Unterschleißheim

2.3 Placental tissue sampling

2.3.1 Sampling of placental tissue for fatty acid analysis

Placental tissue was obtained within 10 minutes after delivery thus minimising metabolic changes in the tissue. Placenta samples were collected from the central parenchyma in each woman, chorionic plate, and areas with pathological abnormalities and/or calcified areas were excluded (Figure 2.1).

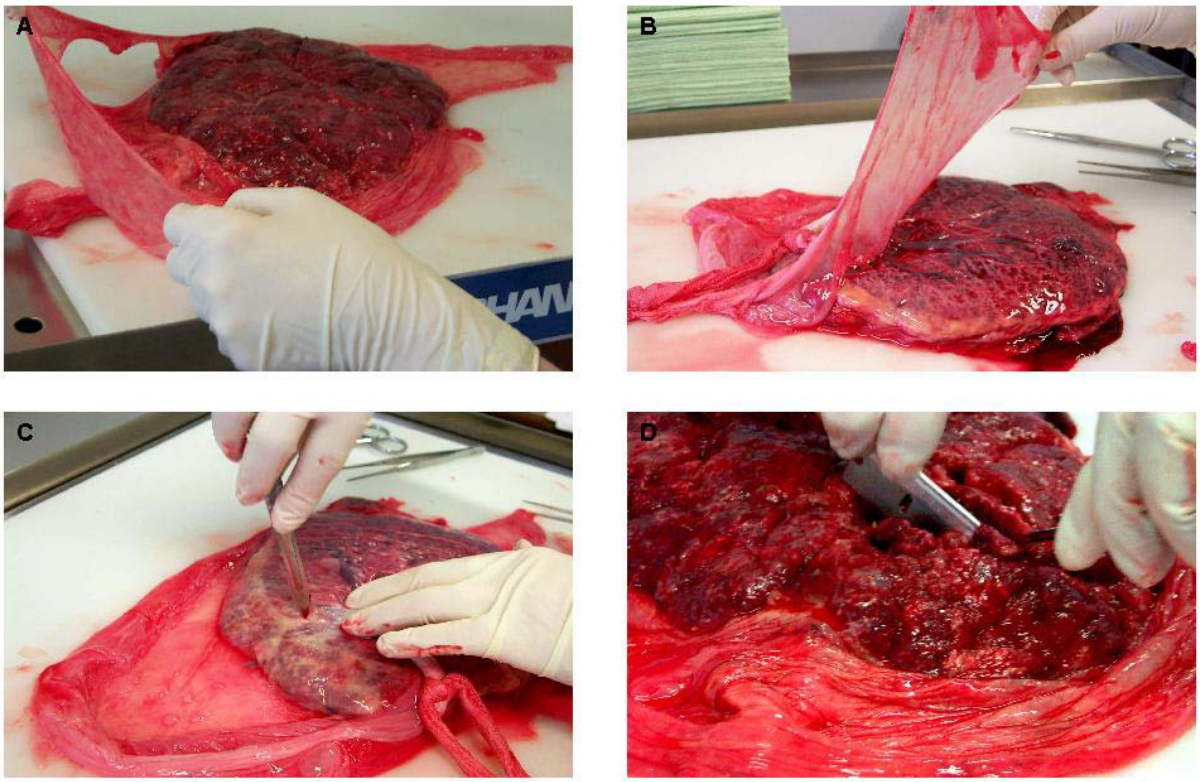


Figure 2.1: Placental sample preparation. View on the basal plate (A) and chorionic plate (B). Connective tissue and chorionic plate were removed (C) and approximately 2 g (0.5x0.5x0.5 cm) were selected for analysis (D).

The pieces (approximately 0.3 g) were washed several times in cold sodium chloride solution (0.9%, 4°C) to eliminate blood residues. Subsequently, it was frozen in liquid nitrogen and stored at –80°C.

2.3.2 Sampling of placental tissue for Western blot

The placental tissue was prepared as described above, but in respect to the heterogeneity of total protein contents in the organ, samples for Western blot were collected

from different locations, which included the centre of parenchyma as well as peripheral locations (Figure 2.2).

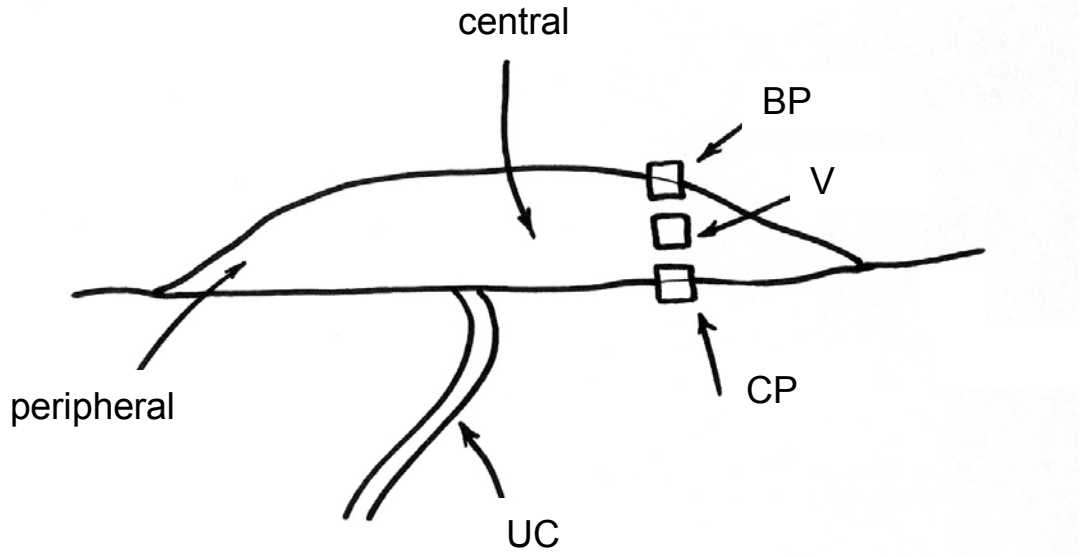


Figure 2.2: Samples for Western blot were collected from different placental locations (peripheral, BP basal plate, V Villi, and CP chorionic plate) and pooled previous to protein analysis. UC umbilical cord.

2.3.3 Sampling of placental tissue for immunohistochemistry

Placental tissue was collected from the central parenchyma as described in 2.3.1, and afterwards to the washing procedure, fixed in neutral buffered 4% paraformaldehyde for 12 h. For dehydrated, the tissue was immersed in gradually increasing concentrations of alcohol to remove the water (starting with 70% alcohol increasing to 100% alcohol). The alcohol in the tissue was replaced by xylene and subsequently embedded in paraffin.

2.4 Fatty acid analysis

2.4.1 Preparation of internal standard

Several approaches were described for internal standards (66;67). C15:0, C17:0 or C19:0 methyl ester were accepted for the quantification of long chain fatty acids (>C14). Also C23:0 or C24:1 were suggested (68). The addition of a single standard assumed no discrimination of individual fatty acids during the processes of extraction, derivatisation and gas liquid chromatography (GLC), furthermore the compensation for any partial hydrolysis that might occur (69). Normally, most of this fatty acids occurred in biological samples, coeluate with other fatty acids in the chromatography or

are hardly soluble (70). Thus, C15:0 methyl ester is a suitable choice as an internal standard (Figure 2.3). It presented good soluble features in polar solvents, it is a minor fatty acid in biological material (<1%) and in spite of short chain length it did not evaporate during sample preparation.

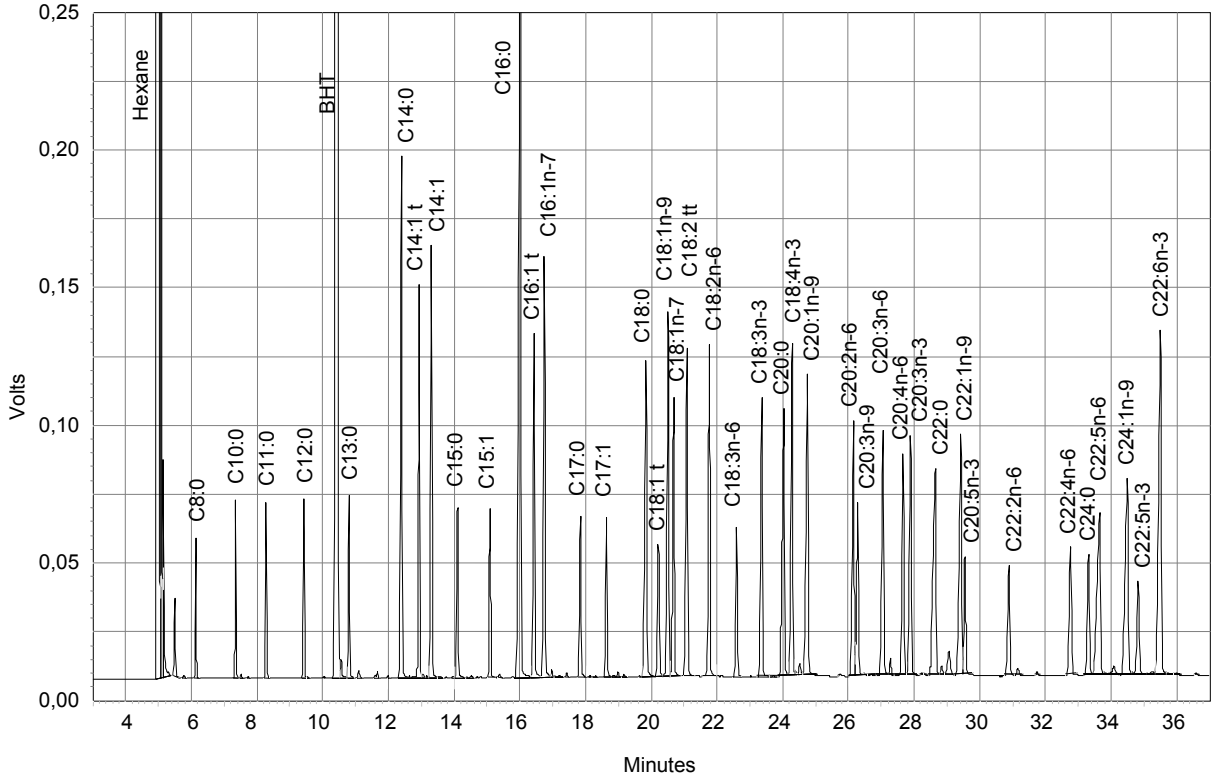


Figure 2.3: Chromatogram of the PL fatty acid profile in a full term placenta (C15:0 internal standard)

For the quantification of fatty acids in PL, NEFA, TG and CE, defined concentrations of phosphatidylcholine dipentadecanoyl, docosapentaenoic acid, tripentadecanoin, and cholesteryl pentadecanoic acid were used. Individual amounts (Table 2.12) were dissolved in 50 ml methanol/chloroform (35:15 v/v).

Table 2.12: Composition of the internal standard in 50ml methanol/chloroform (35:15 v/v)

Lipid	molecular weight [g/mol]	weighted sample [g]
phosphatidylcholine dipentadecanoyl	706,0	5,0
docosapentaenoic acid	242,4	5,2
tripentadecanoin	765,3	12,4
cholesteryl pentadecanoic acid	611	7,3

250 µl of the mixture (≙ 0.025 µg pentadecanoic acid) were added at the beginning of the extraction step to the solution. The concentrations (wet weight) of individual fatty acids were calculated as follow:

$$\text{fatty acid X [mg/ml]} = \frac{\text{peak area (fatty acid X) * standard [mg]}}{\text{peak area (standard) * placental sample weight [g]}}$$

and the proportional contents (wet weight) of individual fatty acids:

$$\text{fatty acid X [\%]} = \frac{\text{fatty acid X [mg/g]}}{\text{total fatty acid content [mg/g]}} * 100$$

Individual fatty acids were identified by comparison with authentic standards (Table 2.6). The elution sequence of the standard mixture is shown in Figure 2.4.

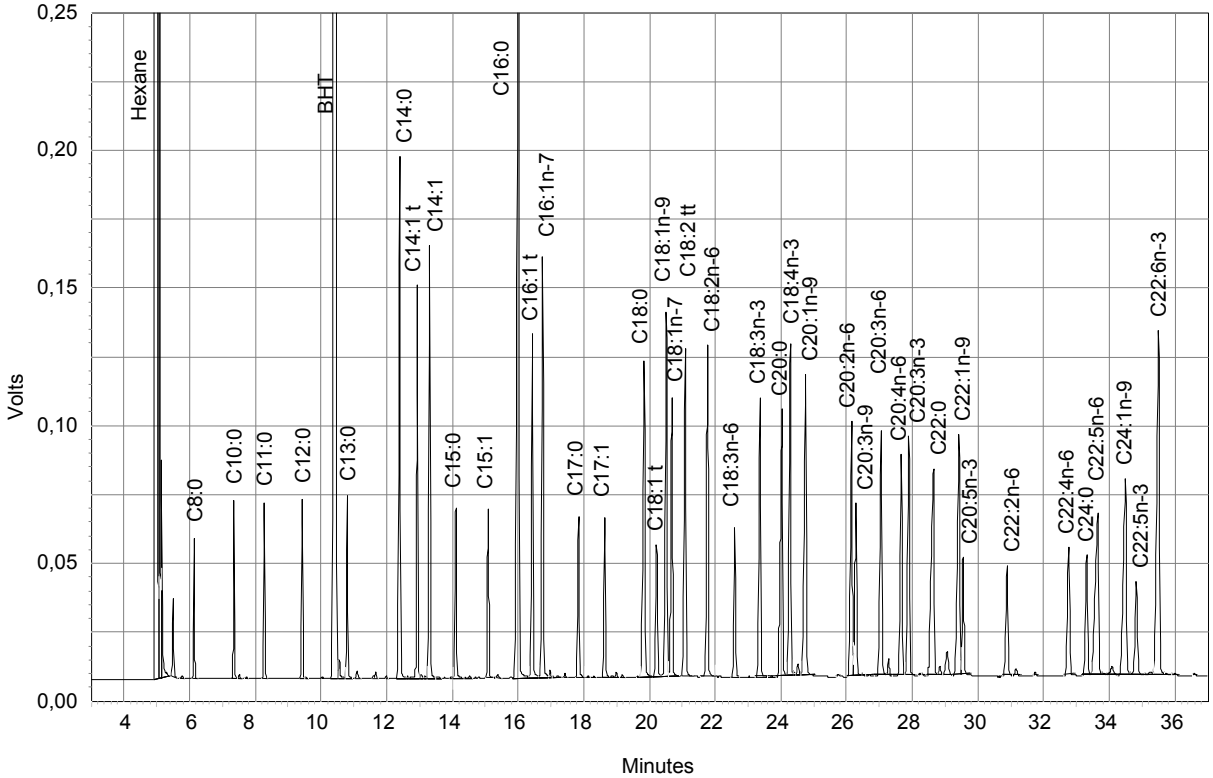


Figure 2.4: Elution sequence and retention times of the standard mixture

2.4.2 Sample preparation

The analysis of fatty acids in biological samples involves mainly three steps: extraction of lipids into an organic solvent (71;72), acid or base catalysed derivatisation to obtain volatile fatty acid methyl esters (FAME) (69;73) and the quantification of individual FAME by gas chromatography with flame ionisation detection (68;74). Until now, there is no validated method for the analysis of the fatty acid content in placental tissue (75-77).

Fatty acids of placental tissue were extracted using a modification of the method of Folch et al. (72). Approximately 0.3 g of tissue was weighed and internal standard was added. Samples were homogenised for 1 min in 12 ml chloroform/methanol mixture (2:1 v/v) with butylated hydroxy toluene (BHT) using a metal-blade homogeniser. Different concentrations (0.5, 2, 3.5, 5, 6 g/l) of BHT were used to avoid oxidation of PUFA during sample preparation. Subsequently, the mixture was heated to 35°C for 20 min, filtered through a glass fibre filter and 4 ml potassium hydroxide solution (0.1 M) were added. The samples were shaken carefully and centrifuged at 900 x g at 10°C for 30 min. Thereafter the aqueous phase was discarded, the organic phase containing the lipids was filtrated over sodium sulfate and taken to dryness under reduced pressure. The remaining lipids were dissolved in 400 µl of chloroform/methanol (1:1 v/v) and carefully deposited on a TLC plate. PL, free cholesterol, NEFA, TG and CE were separated using heptane, diisopropylether and acetic acid (60+40+3, v/v/v) as mobile phase (78). After visualisation of the components with 2´,7´-dichlorofluorescein the bands with the lipid fractions were scraped from the TLC plate and transferred into 4 ml glass tubes equipped with teflon-lined screw caps (69). Methylesters of fatty acids from the fractions were obtained by reaction with 3 M methanolic hydrochloric acid at 85°C for 45 min in closed glass tubes. After neutralisation with sodium carbonate/sodium hydrogen carbonate/sodium sulfate buffer, 1 ml hexane was added. After centrifugation at 400 x g for 3 min the hexane layer was transferred into another vial, the extraction was repeated and the combined extracts were taken to dryness under a gentle stream of nitrogen. Profiling of FAME was performed by capillary GLC, equipped with a Hewlett-Packard 5890 Series 2 gas chromatograph, equipped with a 60 m x 0.32 mm (inside diameter) polar cyanopropyl silicone-coated column (BPX-70). Fatty acid determinations were processed at a column-head pressure of 1.3 bar and an initial temperature of 130°C, followed by an increase of 3°C/min to 180°C, consequently raised to 220°C at 4°C/min.

2.5 Western blot

2.5.1 Positive control (external standard) for the comparison of different blots

For positive control apoptosis was induced in placental explants by incubation with 1.5 mM $MgCl_2$ (79). Small pieces of villous tissue from the centre of placental parenchyma were incubated in medium M199 supplemented with 1% (v/v) fungizone and 1% (v/v) penicillin/streptomycin for 1 hour. After two washings in PBS villi were placed in 1,5 mM $MgCl_2$ DMEM medium, supplemented with 10% (v/v) foetal calf serum, 1% (v/v) fungizone and 1% (v/v) penicillin/streptomycin at 37°C under 5% CO_2 for 48 h (80).

2.5.2 Preparation of placental tissue for Western blot

The samples from different placenta locations were pooled. Approximately 200 mg of placental tissue was boiled in lysis buffer for 5 min, containing 0.01 M Tris (pH 7.4), 1% SDS (w/v), 1 mM Na-Orthovanadate (0.184 g dissolved in 10 ml Tris, pH 10.0) and complete protease inhibitor cocktail. The mixture was homogenised carefully with an metal-blade homogeniser, chilled on ice, centrifuged at 1000 x g at 4°C for 15 min and the aliquots of supernatant was transferred in Eppendorf cups.

2.5.3 Protein analysis

The protein concentrations of all samples was estimated according to Lowry (81). Under alkaline conditions, copper build complexes with protein. When Folin-phenol-reagent is added, the reagent binds to the protein. Bound reagent is slowly reduced and changes colour from yellow to blue.

Lowry's stock reagent:

- Lowry A: 2% Na_2CO_3 in 0.1 M NaOH
- Lowry B: 1% $CuSO_4$ in H_2O
- Lowry C: 2% sodium potassium tartrate ($NaKC_4H_4O_6 \times 4H_2O$)

$\Rightarrow$ Lowry's stock reagent: 49 ml Lowry A + 0.5 ml Lowry B + 0.5 ml Lowry C

Protein concentrations were calculated related to a calibration curve (Figure 2.5). For this approach, bovine serum albumin (BSA) concentrations were dissolved in lysis buffer (10 µl lysis buffer + 90 µl bidest. water, 1:10) (Table 2.13).

Table 2.13: Bovine serum albumin (BSA) concentration for the calibration curve

concentration (µg/µl)	BSA (µg)	lysisbuffer (1:10) (µl)
1	20	980
2	40	960
5	100	900
10	200	800
15	300	700
20	400	600
25	500	500
blanc	-	10

From each of the solutions described in Table 2.13, 10 µl were incubated for 10 min in 250 µl Lowry's stock reagent and subsequently 30 min in 25 µl Folin-phenol reagent (diluted 1:1 with bidest. water before use). The extinction was measured in a spectrometer at 750 nm.

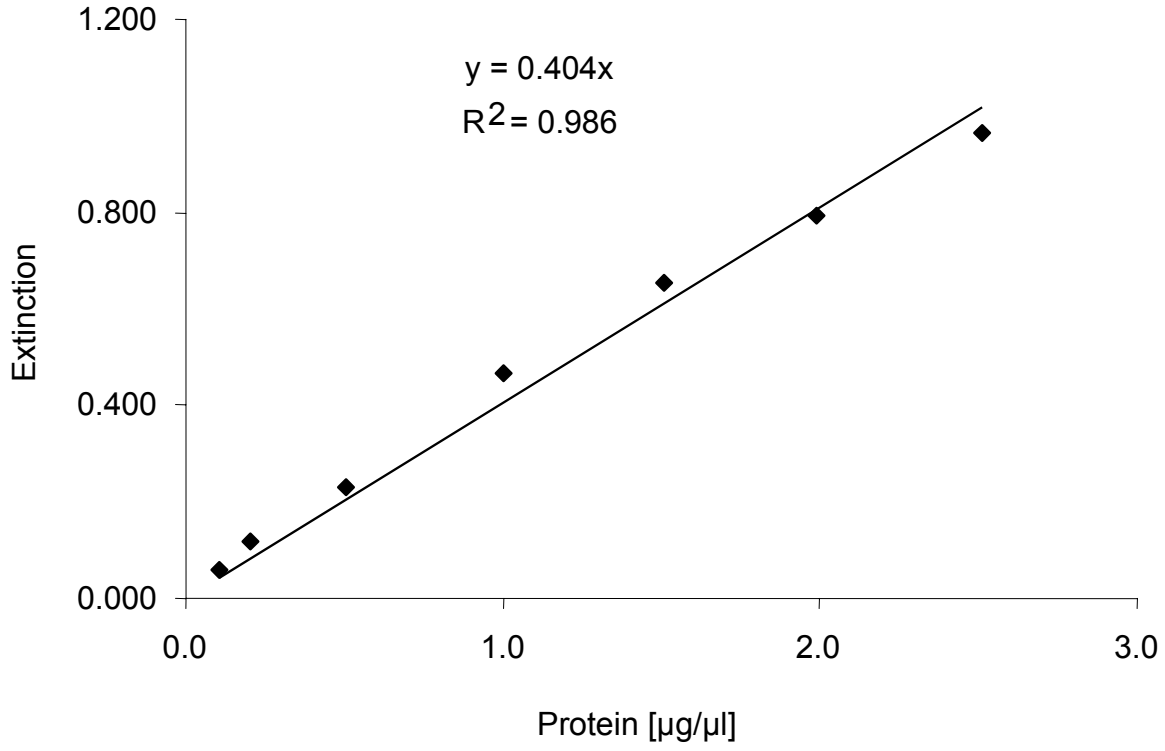


Figure 2.5: Calibration curve for protein analysis in the range of 0.01 to 3.0 µg/µl protein.

The protein concentrations of the placenta samples were calculated as follow:

Protein [µg/µl] = Extinction / 0.404

2.5.4 Western blot analysis

Equal amounts of protein (40 µg per lane) from each sample were used for electrophoresis. The sample solution (25 µl) was calculated and prepared as follow:

sample	x µl
ultrapure water	y µl
sample buffer (4x)	6.25 µl
reducing agent (10x)	2.5 µl
sample solution	25 µl

$$x\ [\mu l] = \frac{40\ \mu g\ (\text{protein per lane})}{\text{Protein concentration } [\mu g/\mu l]}$$

Ultrapure water (y) was added up to 25 µl. The mixture was heated for 10 min at 70°C and subsequently loaded on 4-12% Bis-Tris polyacrylamide gradient gels. The electrophoresis was accomplished under reducing conditions. Afterwards, the proteins were transferred to nitro-cellulose membranes. The membranes were treated with primary antibodies, as listed in Table 2.10, and incubated with a alkaline phosphatase labelled goat-anti-mouse chemiluminescent kit. Incubation times were slightly changed to the manufacturer's protocol (Figure 9.1, p. 81). High performance chemiluminescence films were used for exposure. Times for film exposure and development varied (Table 2.14)

Table 2.14: Film treatment for different antibodies

Antibody	Exposure (min)	Development (min)	Fixation (min)
PCNA	1	1	10
p53	5	1.5	10
Cytokeratin 18 neoepitope	10	1.5	10

Bands corresponding to specific proteins were quantified densidometrically using AlphaEaseFC, Version 3.2.3. For negative control experiments the primary antibodies were exchanged for mouse IgG negative controls. The intensities of the analysed

samples were compared with positive controls and expressed as percentages related to the external standard (arbitrary units).

2.6 Immunochemistry

With a microtome, sections of 5 µm were cut, floated on water and dried at 50°C over night on object slides. The samples were de-paraffinised in xylene and rehydrated in a graded series of alcohol, incubated in 10 mM citrate buffer (pH 6.0) and boiled under pressure in an autoclave at 120°C for 3 min. Unspecific protein binding was blocked with activated human serum (20%) and diluted in V-blocking reagent. Primary antibodies as used for Western blot were diluted in background reducing agent, their binding was detected by biotinylated goat anti-mouse antibody and streptavidin-HRP. After treatment with AEC, slight counterstaining was performed with Mayer's haemalumn (Figure 9.2, p. 82). Slides were mounted with Kaiser's glycerol gelatine. Cells, e.g. trophoblasts, endothelial and other stromal cells, such as blood cells or macrophages, were determined by counting under a light microscope three representative visual fields for each placenta (n=3) per group (original magnification x 400). The low number of samples was considered as sufficient, as the results were verified by Western blot analyses. The proportion of labelled nuclei was expressed to the total number of cells in the field.

2.7 Statistics

For pre-tests, results of fatty acid analysis were expressed as absolute (mg/g wet weight) and as percentage (% w/w) of all detected fatty acids with a chain length of 14-24 carbon atoms. Data of intra- and inter-assay are presented as mean and standard deviation. Time dependent experiments are evaluated using paired t-test.

All data of the intervention study are presented as mean and standard error of mean (SEM). For comparison between groups ANOVA was used and "post-hoc" tests were performed with Bonferroni's correction. Differences between the n-3 LC-PUFA and "non-fish oil" groups were evaluated using unpaired t-tests. Correlation coefficients were calculated according to Pearson. For statistical evaluations SPSS for Windows, Release 11.5.1 was used, with P-values < 0.05 considered statistically significant.

3 Results

3.1 Methodological development of fatty acid analysis

3.1.1 NEFA content at different time points after placenta delivery

If a lipid degradation caused by enzyme activity, e.g. phospholipase A2 (PLA2) or triacylglycerol hydrolyses takes place, the resultant free fatty acids should accumulate in the NEFA fraction. Results indicated a significant increase of the NEFA content after 14.5, 24.5, 34.5, and 44.5 min compared with the initial content (Figure 3.2). Furthermore, accumulation of individual NEFA was shown. The most striking increase was observed for AA, dihomo-γ-linolenic acid and DHA percentages, while percentages of all saturated fatty acids decreased (Table 9.1, p. 84). Therefore, all placental samples were obtained within 10 minutes after delivery.

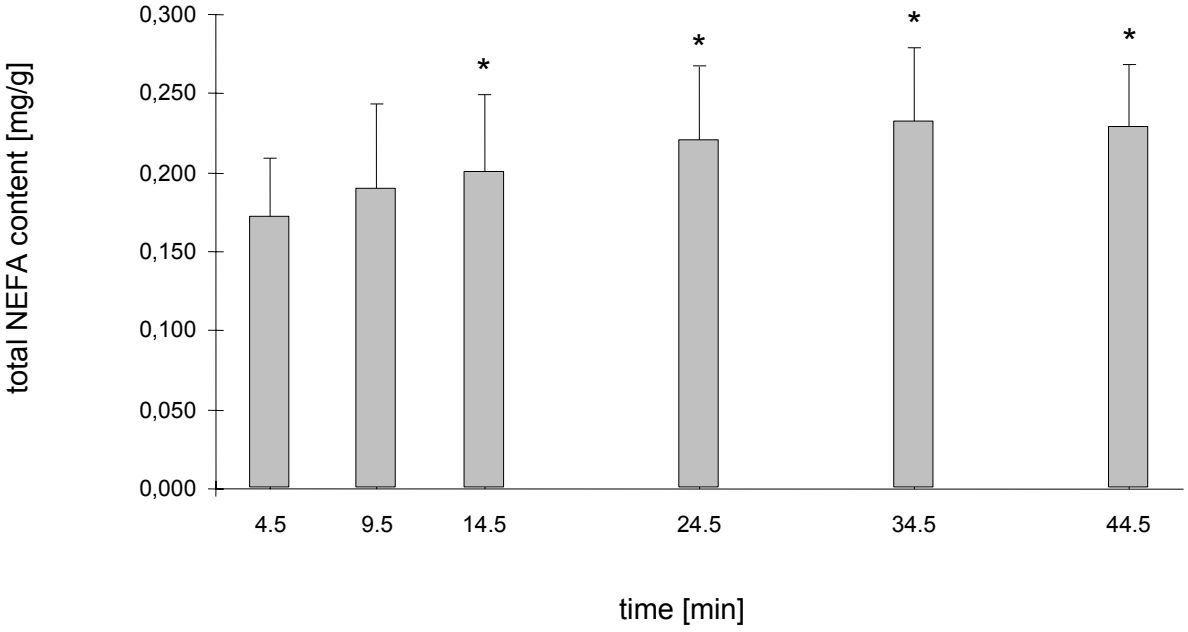


Figure 3.1: NEFA content (mean ± SD) in central placenta parenchyma samples (n=4) over 45 minutes after placenta delivery. Significant alterations are related to the first sample obtained immediately after delivery (4.5 min). *P<0.05.

3.1.2 BHT prevent oxidation of polyunsaturated fatty acids

In order to prevent oxidation of PUFA during sample preparation, BHT at concentrations of 0.5, 2.0, 3.5, 5.0, and 6.0 g/l were added to the chloroform methanol mixture. The variation of the results, expressed by coefficient of variation (CV; n=8), was improved for DHA with increasing concentrations of BHT in the solvent. The CV decreased from 13% (0.5 g/l BHT) to 5.5% (5 g/l BHT) (Figure 3.1).

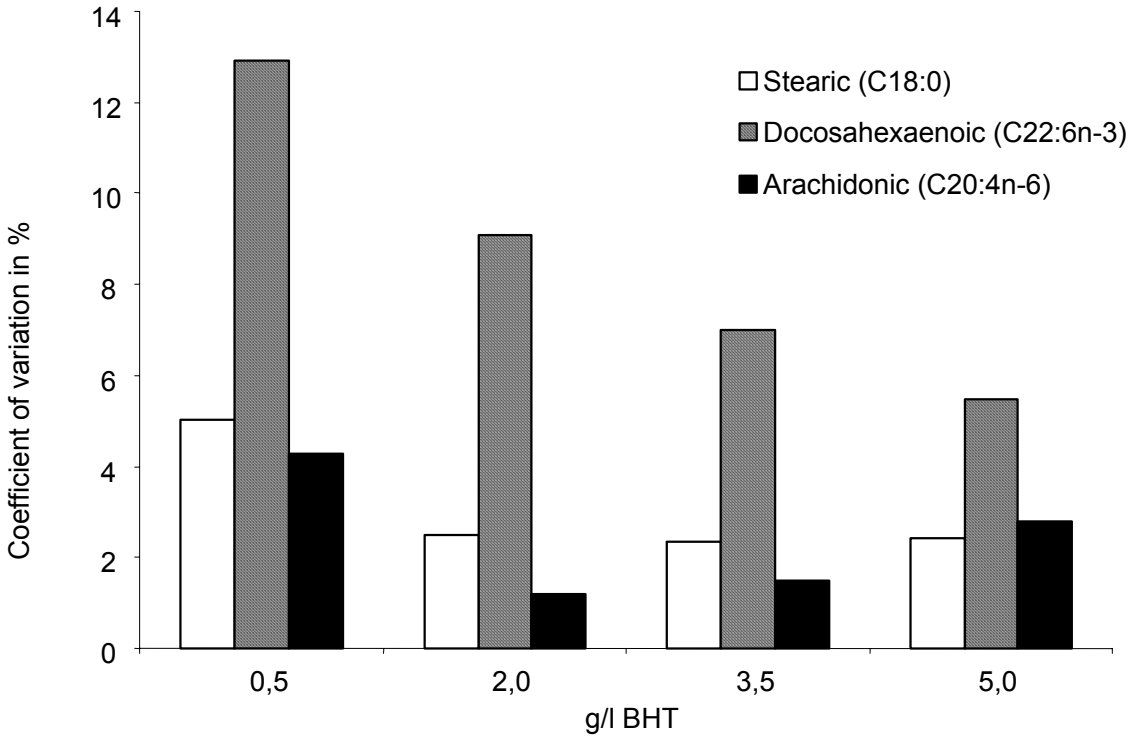


Figure 3.2: Influence of BHT addition to the chloroform methanol mixture for the extraction step. The loss of docosahexaenoic acid decreased depending on the anti-oxidant concentration in the solvent, whereas the other selected fatty acids were hardly influenced (n=8).

The CVs of stearic acid and AA concentrations were lower than 5% in all cases which indicated a small variation and independence from the BHT content. If 6 g BHT per litre were added to the extraction solution, the antioxidant displaced the TG on the TLC-plate leading to a distribution of TG over a larger area and a clear identification of the band under ultraviolet light was no longer possible. Therefore, all further samples were processed with 5g/l BHT in the extraction solution.

3.1.3 Fatty acid content in randomly chosen locations of placental tissue

In order to clarify whether the lipid contents in various parts of the placenta are similar, 2 x 12 randomly chosen samples from two placentas were analysed (Table 9.2, p. 85). The relative composition of the fatty acids with a concentration > 0.01 mg/g in all studied lipid fractions showed a high reproducibility (CV 0.5% - 9.9%) and no systematic pattern was detected. This indicates that the proportional distribution of fatty acids in all locations of the placenta was similar.

The absolute fatty acid concentration in the PL fraction showed a low variation (7.0% - 9.3%) with the exception of docosahexaenoic acid (12.8%). This indicates a homogenous concentration of fatty acids in PL in the whole placenta. However, NEFA,

TG and CE showed differences in the fatty acid content which result in a high variation (CV 6.1% - 42.1%).

3.1.4 Validation of method: intra-assay

The absolute fatty acid content (mg/g) of the different lipid fractions studied in placental tissue are presented in Table 9.3 (p. 86). PL comprised the major part of lipids in placental tissue with 87.5 ± 4.2% (mean ± SD), followed by NEFA (5.7 ± 0.4%), TG (3.8 ± 0.2%) and CE (3.1 ± 0.4%). The detection limit for the analyses was 0.001 mg/g, but the limit of quantification was approximately 0.01 mg/g based on CVs less than 10%. Many of the NEFA, TG and CE fatty acids showed concentration < 0.01 mg/g, and with some exceptions quantification of these fractions was not reproducible. For fatty acid concentrations > 0.01 mg/g, the method indicated a good reproducibility for the PL fatty acids of 4.6% – 11.0% (CV), for NEFA 6.4% – 9.3%, (DHA 13.9%), TG 6.1% - 8.9%, and CE 11.4% - 16.3%. Table 9.4 (p. 87) shows the relative fatty acid composition of the lipid fractions of placental tissue. In PL, NEFA and TG, palmitic acid and AA were the quantitatively most important fatty acids. In contrast, CE showed a different fatty acid pattern with a high abundance of linoleic acid and oleic acid. The CVs for the relative fatty acid composition were for PL 0.4% - 10.5%; NEFA 1.2% - 9.7%; TG 1.8% - 9.0%; CE 2.0% - 7.5%, for fatty acids found in concentrations > 0.01 mg/g.

3.1.5 Validation of the method: inter-assay

In order to determine inter-assay variation, eight tissue samples of one placenta were analysed within a two month period (Table 9.5, p. 88). The reproducibility of fatty acids of the PL and CE fractions was similar compared with the results of intra-assay. However, the fatty acids from NEFA and TG fractions showed higher CVs which might be due to lipolytic alterations during storage.

3.1.6 Determination of the inter-assay during the analyses period

During the analyses period, the inter-assay was determined by samples of one placenta in consistent intervals (one of nine, n=23). By comparison with the inter-assay of the pre-tests, for DHA and AA the reproducibility was slightly improved in PL (CV 5.0% vs. 4.7% and 5.3% vs. 2.1%), NEFA (13.5% vs. 8.7% and 12.1% vs. 7.3%,)

and TG (20.8% vs. 16.5% and 12.3% vs. 11.6%), whereas in CE a higher variation was determined (6.7% vs. 16,4% and 7.2% vs. 8.2%, Table 9.6, p. 89).

3.2 Methodological development of Western blot

3.2.1 Comparison of the intensities of individual lanes

The transfer of proteins from the electrophoreses gel (10 lanes) to the membrane varied per lane. To determine the differences, equal amounts of protein (40 µg) per lane were loaded on the gel and the membrane was incubated with PCNA (n=5). The intensities of lanes 1-9 were compared with lane 10 (Figure 3.3), which was set as 100%. The intensity percentages ranged between 87.5% and 112.5% (lane 1 and lane 5, respectively), therefore for comparisons of different blots, the external standard (see section 2.5.1) was placed on lane 1 and 5. The intensities of the bands of each sample were compared with the mean standard band intensity and expressed as percentages of standard (arbitrary unit).

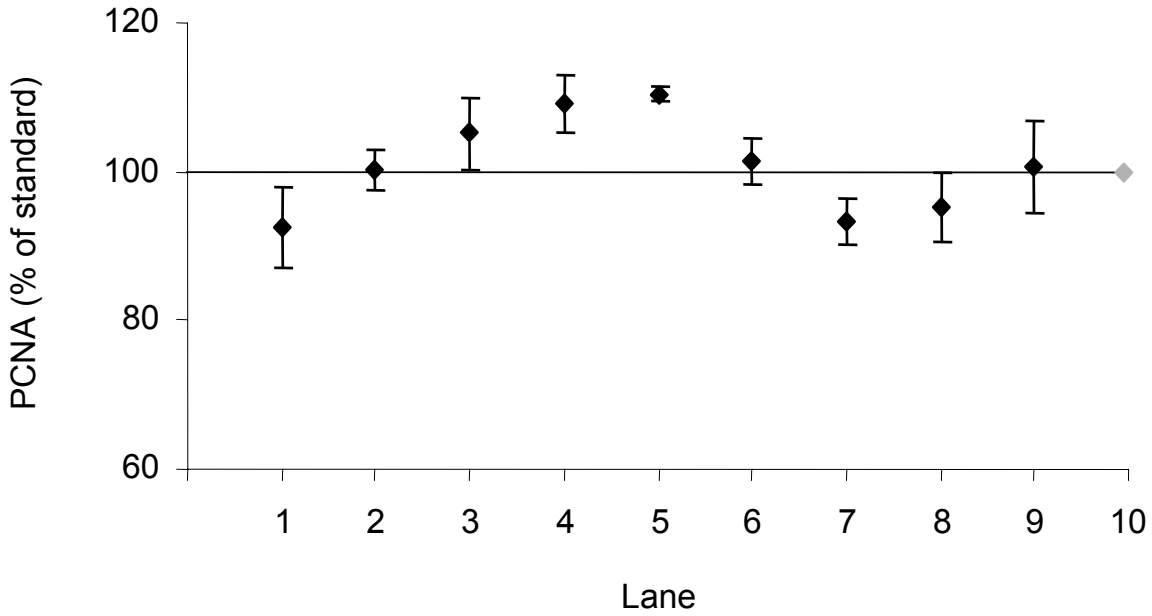


Figure 3.3: Intensity differences of individual lanes compared with lane 10 (mean±S.D., n=5).

3.2.2 Pre-test with selected antibodies

3.2.2.1 Proliferating cell nuclear antigen (PCNA, PC 10)

The antibody PCNA (PC 10) reacts with the PCNA p36 protein expressed at high levels in proliferating cells by Western blotting. It is a marker for cells in early G1 and S phases of the cell cycle. A final dilution of 1:10000 was used for Western blot

analysis (Figure 3.4). In placental tissue an unspecific band at 27 kDa was caused by the second antibody (alkaline phosphatase-conjugated anti-mouse) used by anti-mouse Western Breeze kit (bands were not detected with anti-goat Western Breeze kit).

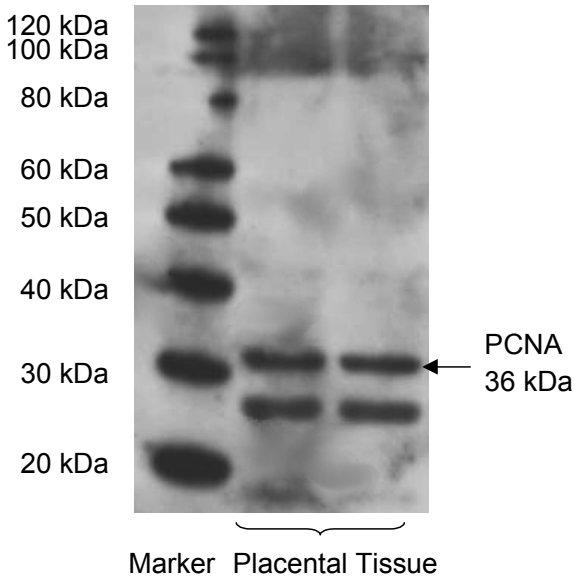


Figure 3.4: PCNA bands of placental positive control cells (external standard) by Western blot (dilution 1:10,000). Bands at 27 kDA were unspecific bands.

3.2.2.2 Tumor suppressor gene p53

The antibody p53 (DO-1) reacts with an amino terminal epitope mapping between amino acid residues 11-25 of wt and mutant p53 of human origin by Western blotting. A final dilution of 1:500 was used for Western blot analysis (Figure 3.5).

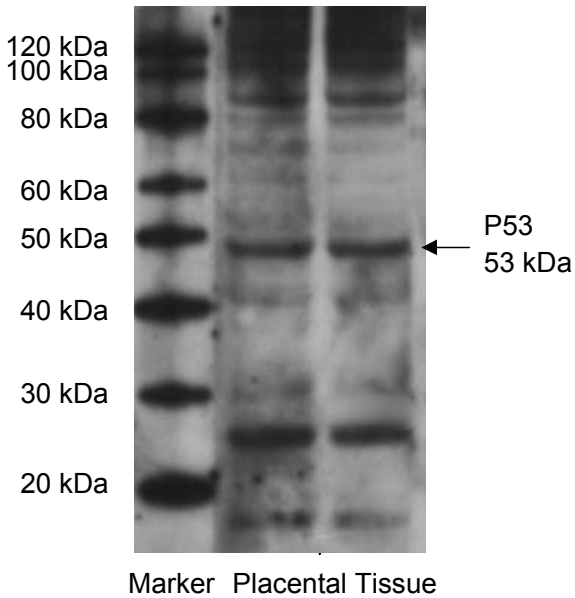


Figure 3.5: P53 bands of placental positive control cells (external standard) by Western blot (dilution 1:500). Bands at 27 kDa were unspecific bands.

3.2.2.3 Cytokeratin 18 neoepitope

The antibody (clone M30) binds to a caspase cleaved epitope of the human cytokeratin 18 cytoskeletal protein. The immunoreactivity of the antibody is confined to the cytoplasma of apoptotic cells. A final dilution of 1:50 was used for Western blot analysis. The method was developed by using a human choriocarcinoma cell line (JAR) established from the trophoblastic tumor of the placenta (Figure 3.6)

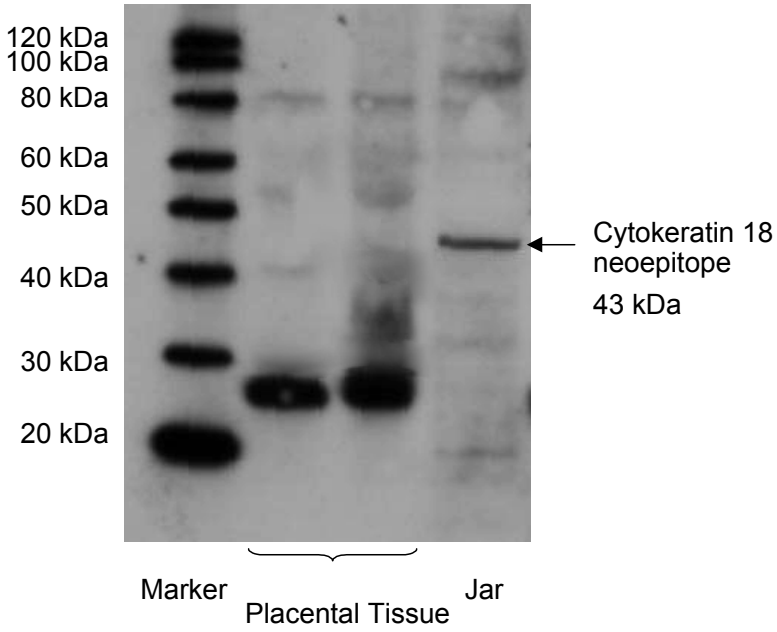


Figure 3.6: Bands of cytokeratin 18 neoepitope in trophoblastic tumor cells (JAR) by Western blot (Dilution 1:50). Bands at 27 kDa in placental tissue were unspecific bands, in the culture cells (JAR) such bands were not detected.

3.3 Intervention study

3.3.1 Placental fatty acid profile

3.3.1.1 Subjects

A total of 302 pregnant women were recruited for the study, 224 participated until giving birth. All placentas of these women were scheduled for fatty acid analysis in the lipid fractions. 22 placental samples could not be analysed, because they were to small for fatty acid analysis or tissue was not collected. Thus, 202 placental samples were available for fatty acid determination. Significant differences between the population from the three study centres were observed for length of gestation and maternal age (Table 3.1).

Table 3.1: Characteristics of study population

	Spain* (n=100)	Germany** (n=48)	Hungary (n=54)
Length of gestation (d)	276,8 (1,1)[c]	275,5 (2,2)[b]	266,8 (2,0)[b,c]
Maternal age (y)	30,3 (0,5)[a]	33,7 (0,5)[a,b]	29,8 (0,7)[b]
BMI at study entry (kg/m^2)	25,6 (0,4)	24,8 (0,5)	25,5 (0,6)
BMI at delivery (kg/m^2)	29,5 (0,5)	28,8 (0,6)	29,0 (0,6)
Placental weight (g)	549 (14)	554 (17)	519 (12)
Infants' birth weight (g)	3275 (47)	3369 (79)	3270 (71)
Infants' birth length (cm)	50,6 (0,2)	52,0 (0,5)	49,5 (0,4)
Smoking during pregnancy (%)	18	4,2	1,8

* Not all data were available for the Spanish population: BMI at study entry (98), BMI at delivery (92), placental weight (97), birth weight (99) and birth length (91). ** The German placental weights were available for 47 subjects. Identical superscripts indicate significant differences between: a) Spain and Germany, b) Germany and Hungary, c) Spain and Hungary, $p < 0.05$.

On average, the Hungarian pregnancies were approximately ten days shorter than in the other countries. Pre-term deliveries or prolonged gestations were not excluded, because restrictions to deliveries only at term (37-42 weeks of pregnancy) did not significantly change the average placental PL fatty acid pattern of our study population. The mean age of German mothers was approximately four years higher than in the other countries. No significant differences between centres were observed for maternal BMI, placental weight and the anthropometric measures of the infants (Table 3.1).

3.3.1.2 Relationship between the habitual diet and the placental PL fatty acid profile

The fatty acid composition in placental PL in German, Spain and Hungarian women not consuming supplemented n-3 fatty acids or 5-MTHF are listed in Table 9.7 (p. 90). The total content of n-6 and n-3 fatty acids in PL was similar in German and Spanish women. The Hungarian placentas contained significantly less DHA than those from the other countries and also showed significantly lower total n-3 fatty acid contents. Total amounts of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), the trans fatty acids and n-9 polyunsaturated fatty acids (PUFA) were not different between the three countries.

3.3.1.3 Supplementation effects on the fatty acid pattern of placental PL

The supplementation effects were compared in the whole study population (Table 9.8, p. 91). The additional intake of the n-3 LC-PUFA increased DHA, EPA and total

of n-3 fatty acids in PL ($p<0.05$). The AA content and the total n-6 fatty acids were not affected by the supplementation, which led to lower n-6/n-3 ratios. The additional intake of DHA and EPA did not influence the ratio of the n-3 to n-6 fatty acid precursors linoleic acid (LA, 18:2n-6) to α-linolenic acid (ALA, 18:3n-3), respectively, as well as the ratio of AA (20:4n-6) to dihomo-γ-linolenic acid (DGLA, 20:3n-6), which may reflect Δ^5-desaturase activity. The DHA/EPA ratio (22:6n-3/20:5n-3) was not significantly different between groups, but the supplemented groups tended to show lower quotients. AA/EPA and AA/DHA rations reflected the additional DHA and EPA intake, resulting in lower ratios.

No significant differences were found between the placebo and the 5-MTHF supplemented group as well as for the n-3 LC-PUFA and the n-3 LC-PUFA / 5-MTHF supplemented group for each country. There were no significant differences between the countries for the supplemented groups, except for the placebo group, where the DHA contents in Hungarian placental PL were significantly lower compared with German and Spanish placentas. The Hungary placebo group indicated significantly lower DHA contents, whereas the supplemented group with n-3 LC-PUFA or the combination showed similar levels to the supplemented groups from Germany and Spain (Figure 3.7).

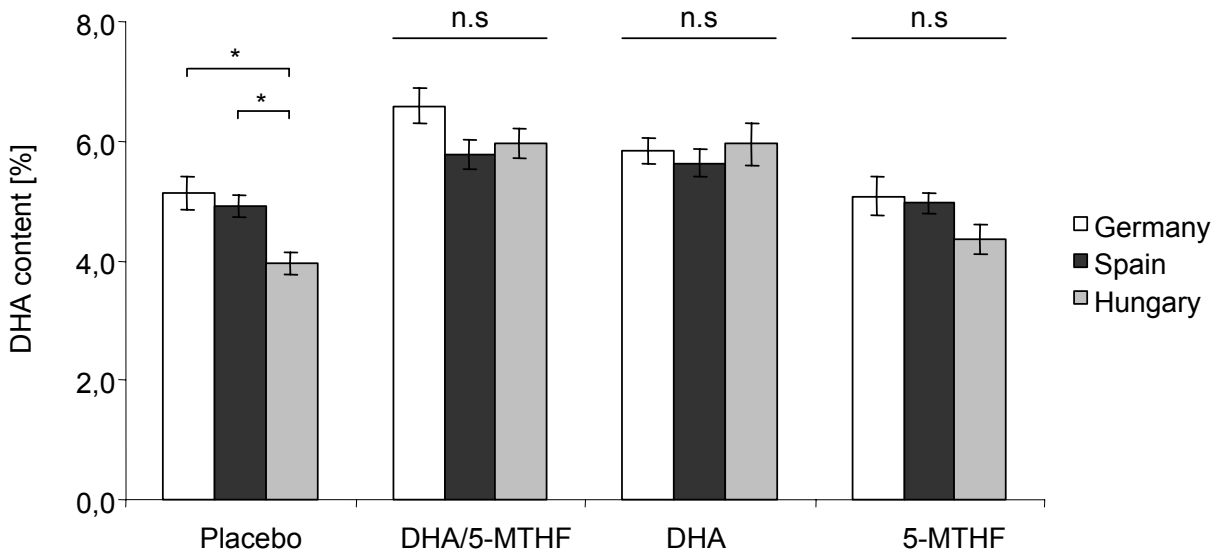


Figure 3.7: Comparison of the DHA and 5-MTHF supplementation in placental phospholipids in Germany, Spain and Hungary. n.s no significance within the supplied group and the different study populations. *P < 0.05.

3.3.1.4 Supplementation effects on the NEFA, TG and CE fatty acid pattern

As described above, 5-MTHF did not significantly change the fatty acid profile of placental PL, thus for further investigation, the n-3 LC-PUFA treated group and the group which received both n-3 LC-PUFA and 5-MTHF were combined to the n-3 LC-PUFA group and the groups which received the placebo or the 5-MTHF supplement were combined to the control group.

In the whole population the DHA contents were significantly higher in placental PL, NEFA, TG and CE in the n-3- LC-PUFA treated group as compared with the placebo group (5.88±0.11% vs. 4.77±0.10%, 5.28±0.15% vs. 4.12±0.13%, 6.80±0.33% vs. 4.64±0.22%, and 3.98±0.14% vs. 3.00±0.10%, respectively, Figure 3.8).

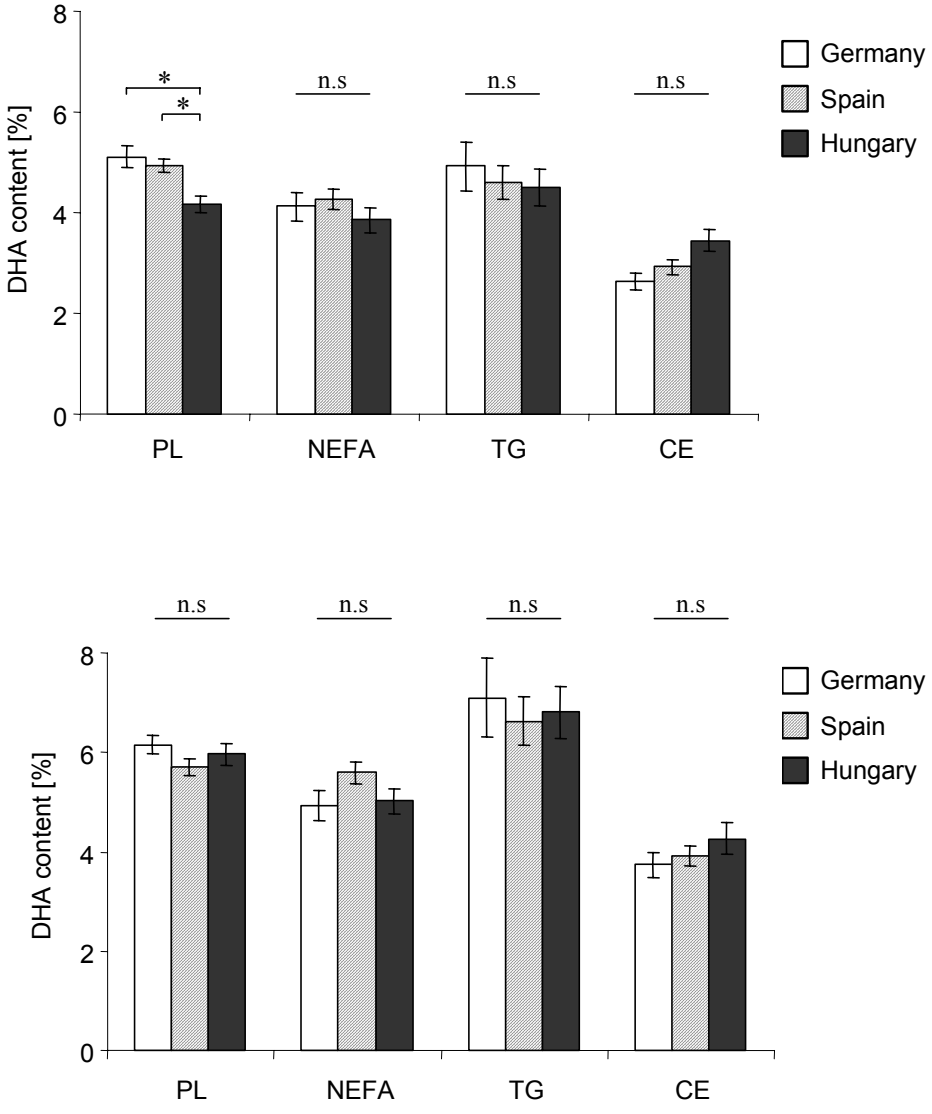


Figure 3.8: Comparison of the placental lipid fraction docosahexaenoic acid (DHA) contents between the placebo group (A) and the n-3 LC-PUFA supplemented group (B) (*p<0.05).n.s. not significant.

Furthermore, the low EPA content (approximately 150 mg) in the supplement increased also the EPA content in any placental lipid fraction. The total n-3 LC-PUFA content in all lipid fractions was significantly enhanced in the n-3 LC-PUFA treated group. The additional uptake of n-3 LC-PUFA did not change the proportions of other fatty acids, with exception of n-6 docosapentaenoic acid (n-6 DPA, C22:5n-6), which was higher in the placebo group compared with the supplemented group (PL 1.11%±0.04% vs. 0.80±0.01%, NEFA 0.95±0.04% vs. 0.68±0.03%, TG 1.18±0.06% vs. 0.86±0.04% and CE 1.03±0.05% vs. 0.77±0.04%). Therefore, the ratio of n-3 to n-6 polyunsaturated fatty acids (PUFA) in the n-3 LC-PUFA treated group was lower in all lipid fractions ($p<0.05$), whereas the ratio of PUFA to saturated fatty acids (SFA) was not affected (Table 9.9, p. 92).

To test the potential relation of AA and DHA compositions between NEFA, TG and PL, correlation analyses between percentages of individual values were performed. Correlation between NEFA and PL ($r^2 = 0.003$) were not significant for AA, whereas correlation was significant for DHA ($r^2 = 0.506$; $P<0.01$). Correlation between NEFA and TG were significant for AA ($r^2 = 0.110$; $P<0.01$) and DHA ($r^2 = 0.302$; $P<0.01$, Figure 3.9 and 3.10).

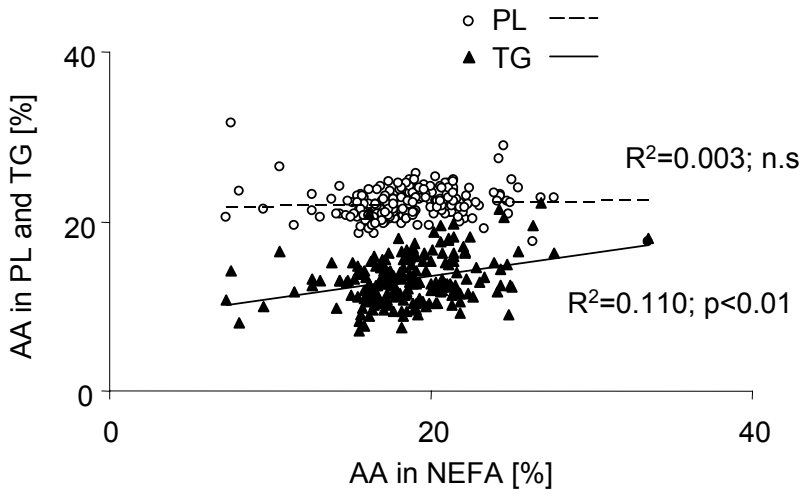


Figure 3.9: Dependence of AA percentages in PL and TG from their corresponding proportion in the NEFA pool (n=202). n.s not significant.

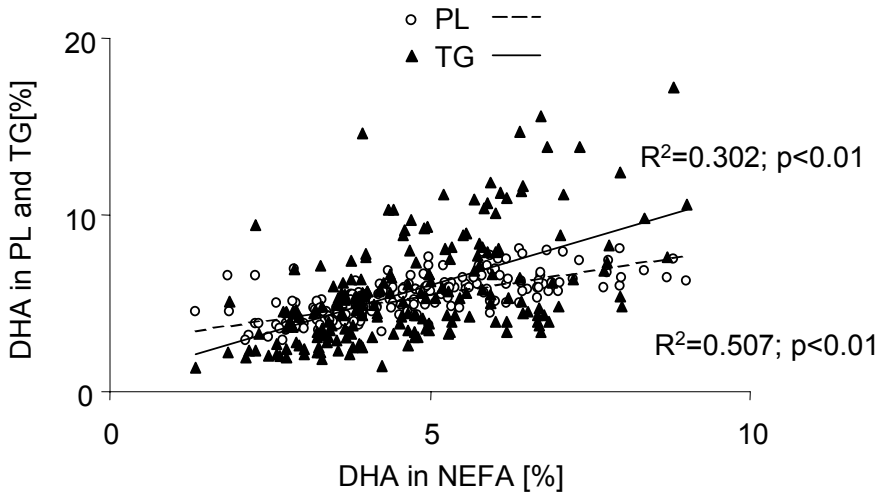


Figure 3.10: Dependence of DHA percentages in PL and TG from their corresponding proportion in the NEFA pool (n=202).

The AA and EPA content was negatively correlated in PL (r^2=0.069; P<0.01) and in CE (r^2=0.069, P<0.01), whereas no correlations in NEFA (r^2=0.016; P>0.05) and in TG (r^2= 0.004, P>0.05) were found (Figure 3.11).

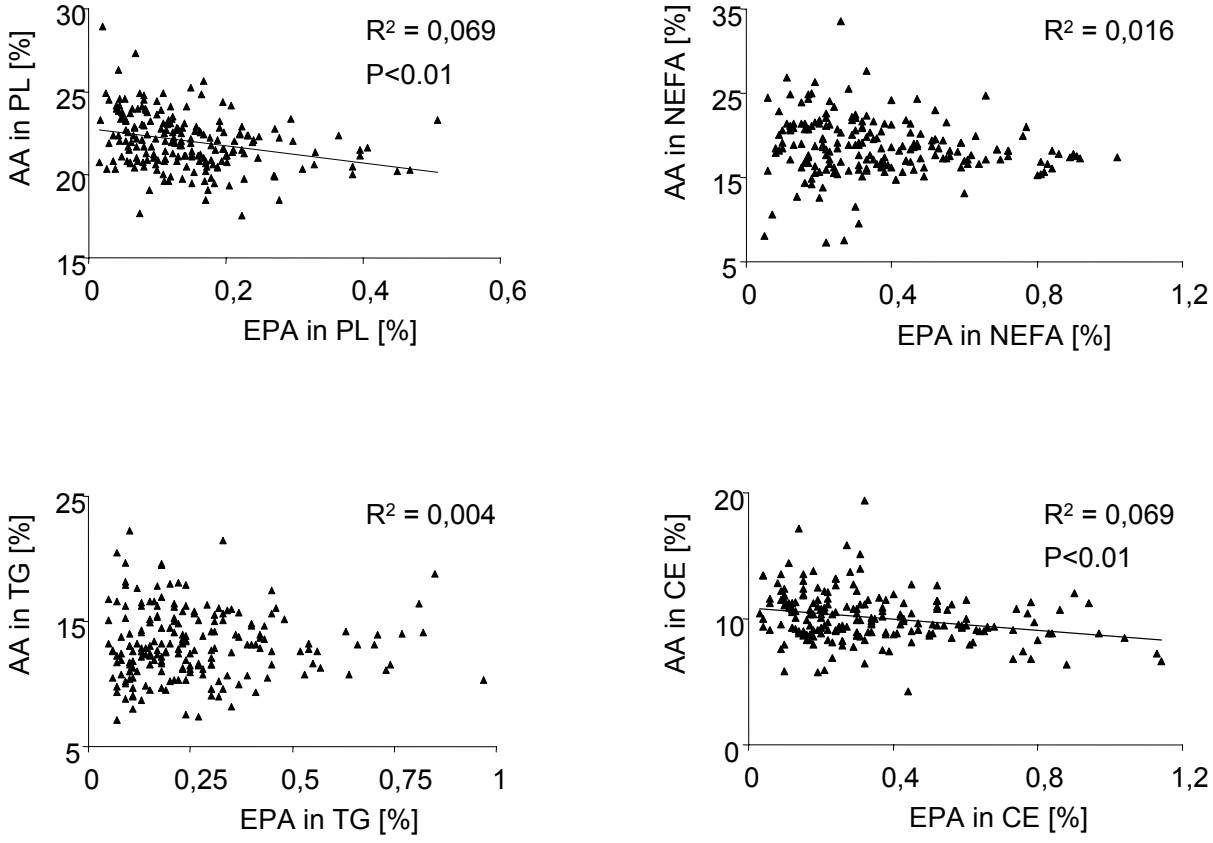


Figure 3.11: Relationship between eicosapentaenoic acid (EPA) and arachidonic acid (AA) in different placental lipid fractions (n=202).

The comparison of the placental lipid fractions without differentiating between the three centres showed that the supplementation with n-3 LC-PUFA did not alter the concentrations of individual fractions: PL 7.36±0.11 mg/g wet weight vs. 7.44±0.12 mg/g, NEFA 0.30±0.01 mg/g vs. 0.31±0.01 mg/g, TG 0.17±0.01 mg/g vs. 0.18±0.01 mg/g, and CE 0.42±0.02 mg/g vs. 0.40±0.02 mg/g (placebo group vs. n-3 LC-PUFA group). Therefore, the contribution of the fractions to the total lipid content was not different between the groups: PL 89.3±0.3% vs. 89.3±0.3%; NEFA 5.0±0.2% vs. 4.7±0.2%, TG 3.7±0.1% vs. 3.7±0.1%, and CE 2.0±0.1% vs. 2.2±0.1% (placebo group vs. n-3 LC-PUFA group).

3.3.2 Western blot and Immunohistochemistry

3.3.2.1 Subjects

Placental apoptosis and proliferation was determined in a subgroup of the Spanish population. Out of the total of 154 studied women, eight did not finish the clinical trial. The sample collection was limited due to logistic restrictions, thus 105 samples were available for fatty acids analysis. For apoptosis and cell cycle analyses only 91 pieces of placental tissue were collected in the strict random sampling mode described above. Eleven of the samples were too small for the analyses of all parameters. The full set of parameters was investigated for 54 women. Characteristics of the study population are given in table 3.2.

Table 3.2: Characteristics of the study population, mean (SEM). No statistically differences were found between the groups.

	supplement			
	placebo (n=12)	fish oil / folate (n=11)	fish oil (n=16)	folate (n=15)
Length of gestation (d)	281.5 (2.0)	274.0 (3.9)	279.4 (2.3)	276.1 (9.8)
Maternal age (y)	31.6 (0.8)	30.8 (1.3)	28.1 (1.3)	29.0 (1.3)
BMI at study entry (kg/m^2)	25.2 (1.1)	25.8 (0.9)	25.2 (1.1)	25.1 (1.0)
BMI at delivery (kg/m^2)	29.7 (1.5)	29.1 (0.8)	29.1 (0.5)	27.6 (2.5)
Placental weight (g)	586 (24)	551 (52)	553 (34)	527 (41)
Infants' birth weight (g)	3316 (71)	3110 (191)	3288 (127)	3215 (129)
Infants' birth length (cm)	51.0 (0.6)	49.9 (0.7)	50.6 (0.5)	50.9 (0.6)
Smoking during pregnancy (%)	0	27.3	12.5	16.6

3.3.2.2 Results of the fatty acid analyses of the subgroup

The fatty acid patterns of the evaluated 54 subjects are representative for the total collective. Fatty acid compositions in the subgroup were determined in placental PL, NEFA and TG fractions. The individual fatty acids composition showed no differences between the four treatment groups, with the exception of the supplemented fatty acids, DHA and EPA (Figure 8.1). The proportion of DHA in the placental PL indicated similar contents for n-3 LC-PUFA treated groups (n-3 LC-PUFA 5.9±0.3%, combined 6.2±0.3%), which were different ($p<0.05$) to "non-fish oil" supplemented groups (placebo 4.8±0.2%, 5-MTHF 4.9±0.2%). Differences ($p<0.05$) were also measured for EPA (n-3 LC-PUFA 0.2%±0.0 and combination 0.3±0.0% vs. placebo 0.1±0.0% and 5-MTHF 0.1±0.0%). In the cytosolic NEFA pool EPA contents differed ($p<0.05$) between placebo group (0.4±0.1%) and n-3 LC-PUFA / 5-MTHF group (0.6±0.1%). This was also observed for the DHA contents in the TG fractions (combination group 7.9±0.7%, 5-MTHF group 5.2±0.5%), furthermore differences ($p<0.05$) were indicated for EPA contents between placebo (0.2±0.0%) and n-3 LC-PUFA (0.6±0.1%) supplementation. The additional uptake of EPA could displace the arachidonic acid in the different lipid fractions, respectively, in the present study this effect was not observed (Table 3.3).

Table 3.3: Contents of arachidonic acid in different placental lipid fractions (w/w in %).

	Supplement			
Lipid fraction	Placebo (n=12)	fish oil / folate (n=11)	fish oil (n=16)	folate (n=15)
PL	21,2 ± 0,4	21,8 ± 0,4	21,3 ± 0,4	22,2 ± 0,3
NEFA	17,6 ± 0,7	17,9 ± 0,5	18,5 ± 0,5	18,0 ± 0,6
TG	12,6 ± 0,5	12,3 ± 0,6	12,9 ± 0,6	12,1 ± 0,8

3.3.2.3 Folate concentrations

The folate concentration in maternal and foetal blood of 142 available samples were analysed by the Department of Clinical Medicine, Trinity College Dublin. The data showed that the evaluated subgroup (n=54) are representative for the total population. The additional intake of folate was reflected in maternal blood plasma concentrations, i.e. the folate supplemented groups (folate 12.6±1.3 ng/ml and combination 16.0±1.8 ng/ml) indicated higher ($p<0.05$) contents compared with the untreated

groups (placebo 4.5±0.8 ng/ml and n-3 LC-PUFA 6.6±1.1 ng/ml). Contrary, in foetal plasma no differences were found (Figure 3.12). The ratio of the plasma folate concentration between mother and child decreased significantly after 5-MTHF supplementation (folate 1.2%±0.1 and combined 1.3%±0.1 vs. placebo 4.2%±0.9 and n-3 LC-PUFA 3.0%±0.3).

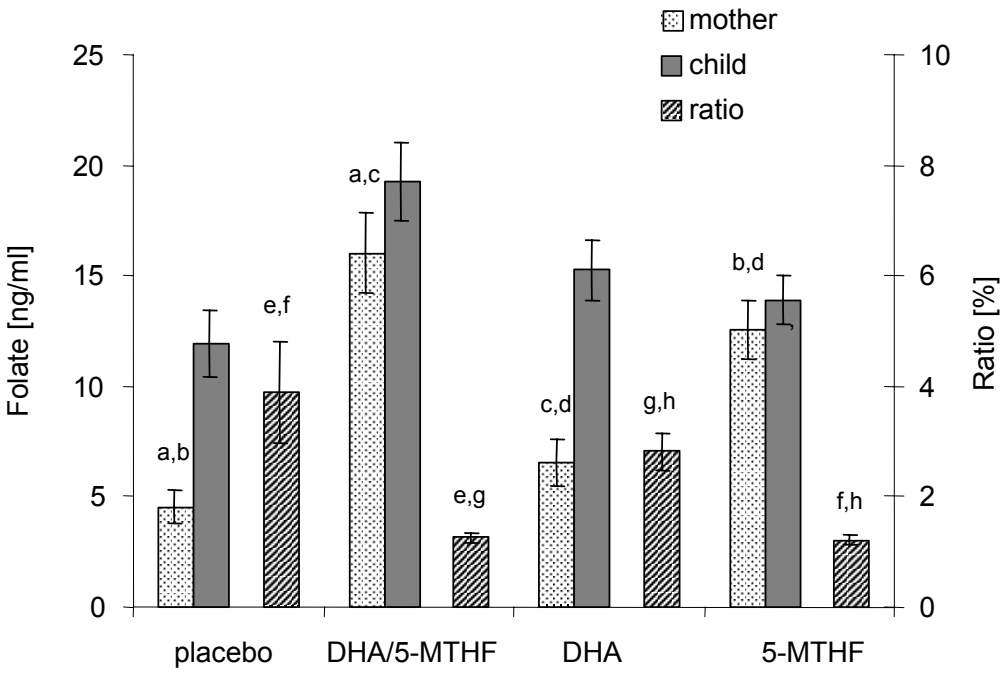
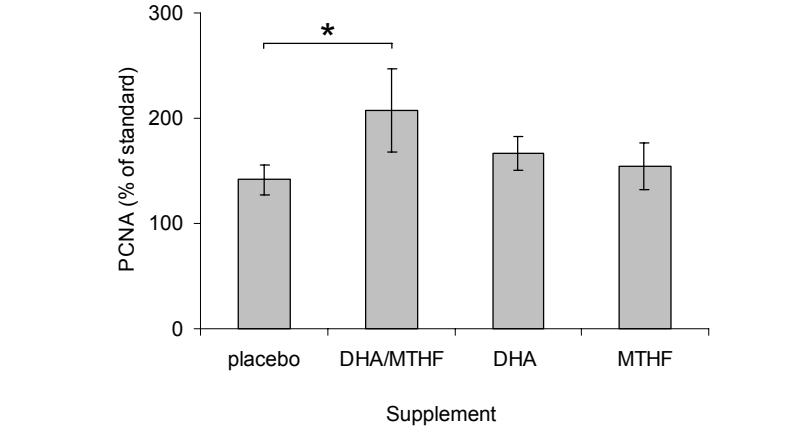


Figure 3.12: Folate supplementation effect on foetal and maternal plasma folate concentrations. In maternal plasma higher concentrations are measured in the 5-MTHF supplemented groups. In foetal plasma no differences are found between the four groups. Ratio of maternal and foetal plasma folate concentration was reduced by additional 5-MTHF uptake. Identical superscripts indicated significant differences. (*p<0.05).

3.3.2.4 Western blot and immunohistochemistry

The proliferation marker PCNA, the tumor suppressor p53, and the caspase cleavage product of cytokeratin 18 (clone M30, apoptosis marker), were immunolocalised to determine proliferation and apoptosis in term placenta. The expression of PCNA in the DHA / 5-MTHF treated group was higher in 66.3% ($p<0.05$) compared with the placebo group (Figure 3.12). Cytokeratin 18 neoepitope and p53 levels by western blot were unaffected by treatment (Figures 3.13 and 3.14).



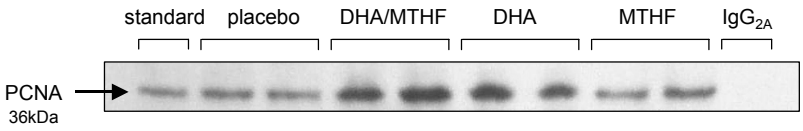
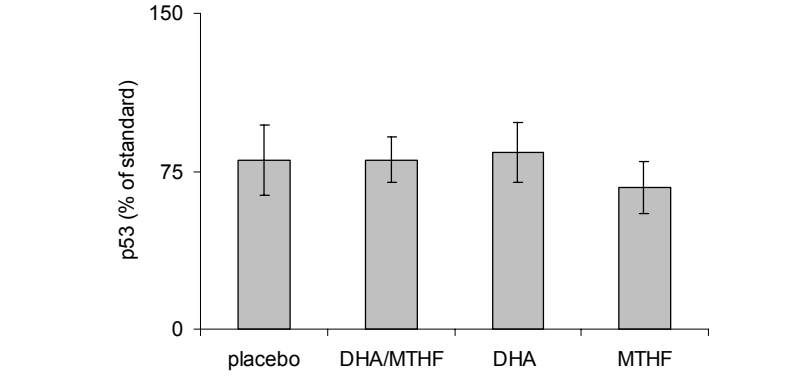


Figure 3.13: Immunoblot and densitometric analysis of the protein bands of PCNA



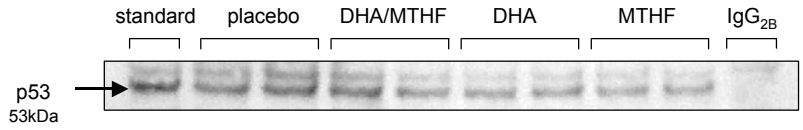


Figure 3.14: Immunoblot and densitometric analysis of the protein bands of cytokeratin 18 neoepitope (clone M30)

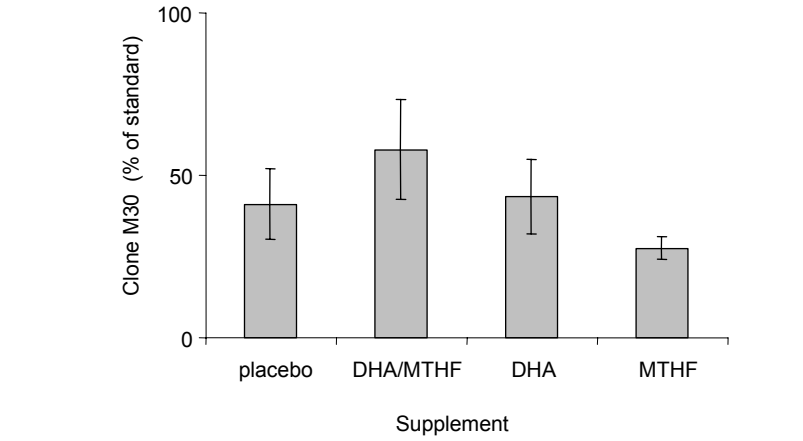


Figure 3.15: Immunoblot and densitometric analysis of the protein bands of p53

The Western blot results were confirmed by immunohistochemical analyses (Figures 3.16, 3.18 and 3.19). The proportion of cells of PCNA was determined for the DHA/5-MTHF treated group and the placebo group (Figure 3.16).

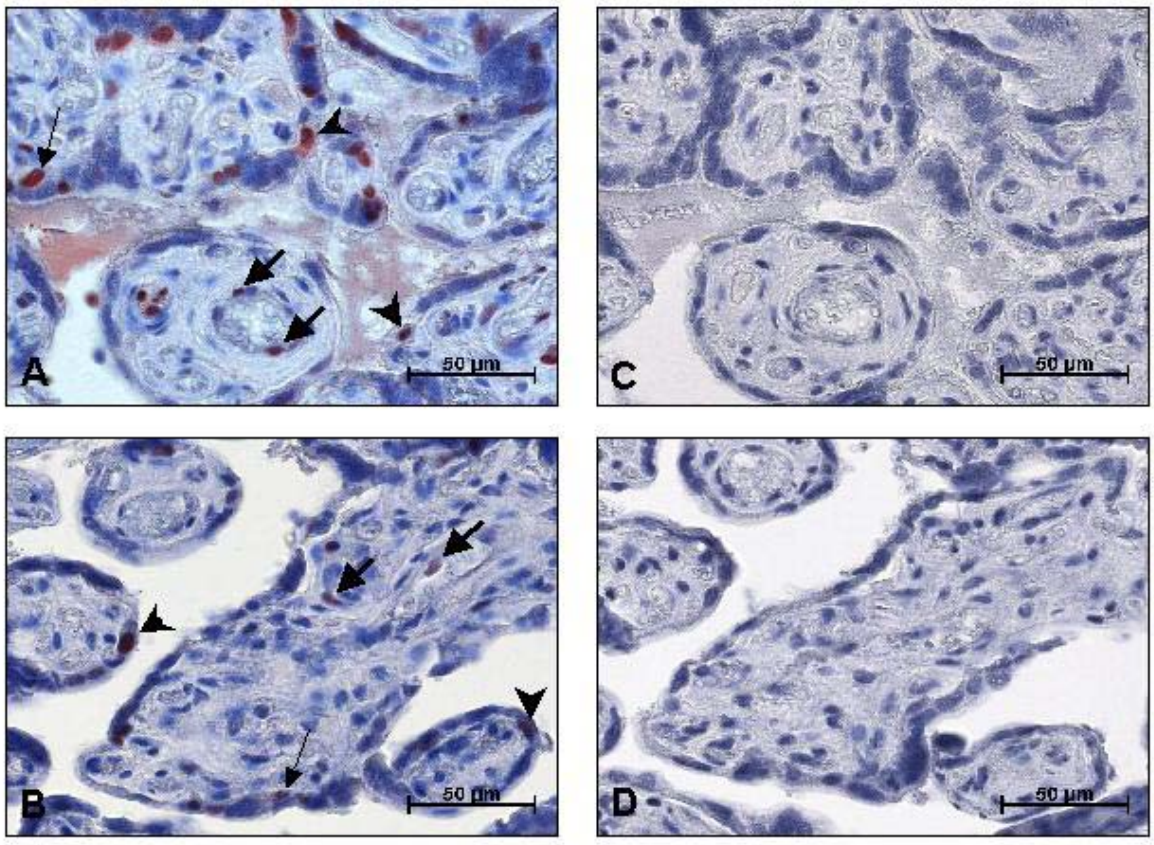


Figure 3.16: Immunolocalisation with antibody PCNA (proliferation) in full term placenta. PCNA immunostaining was stronger in placental tissue of the n-3 LC-PUFA/5-MTHF supplemented group (A) then in the placebo group (B). PCNA in endothelium and other cells showed no differences between both groups. Panels C and D shows IgG isotype matched negative controls from a parallel section. black arrowhead: syncytiotrophoblast, thin arrow: cytotrophoblast, bold arrow: endothel and other cells.

The proportions of nuclei labelled for PCNA were higher ($p<0.05$) in the combination supplemented group (12.8%) compared with the placebo treated group (7.3%) in the trophoblast compartment, which was the result of changes in total, whereas similar proportions of nuclei was stained in endothel and other stromal cells (Figure 3.16).

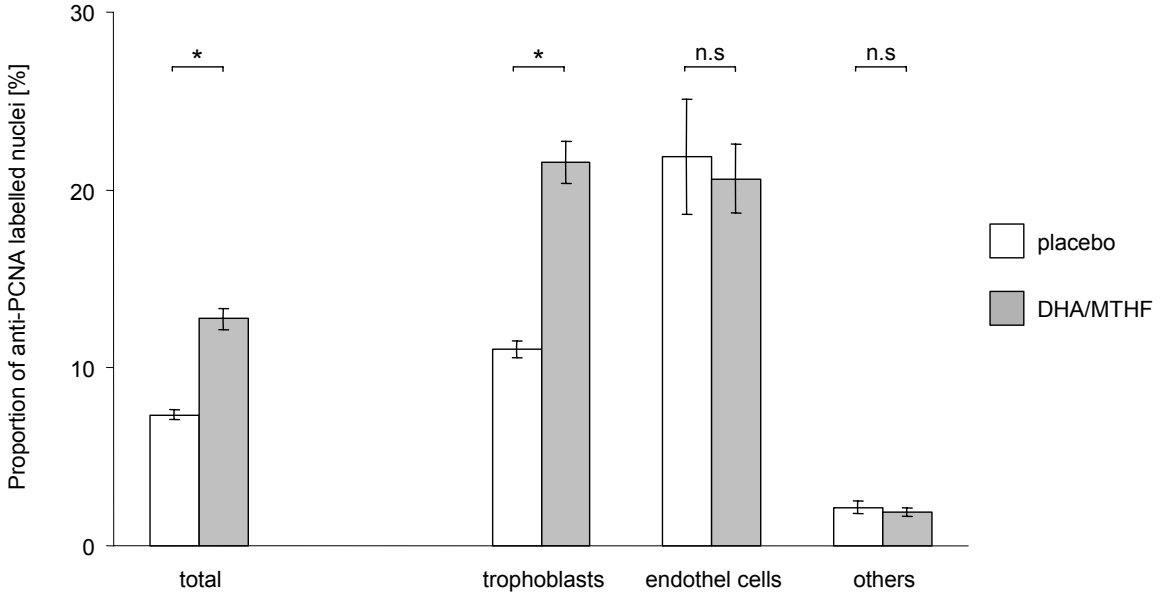


Figure 3.17: A higher proportion of nuclei were immunolabelled in the n-3 LC-PUFA / 5-MTHF (n=3) group as compared to placebo (n=3), because of an exclusive increase in the trophoblast compartment. The labelling of endothel cells and other cells, such as blood cells and macrophages, remained unchanged. (*p<0.05; n.s: not significant).

The staining intensity of p53 positive nuclei was similar in all four study groups (Figure 3.17), which is in agreement with the similar apoptosis rates, defined by were found almost exclusively among extra villous trophoblasts (Figure 3.18).

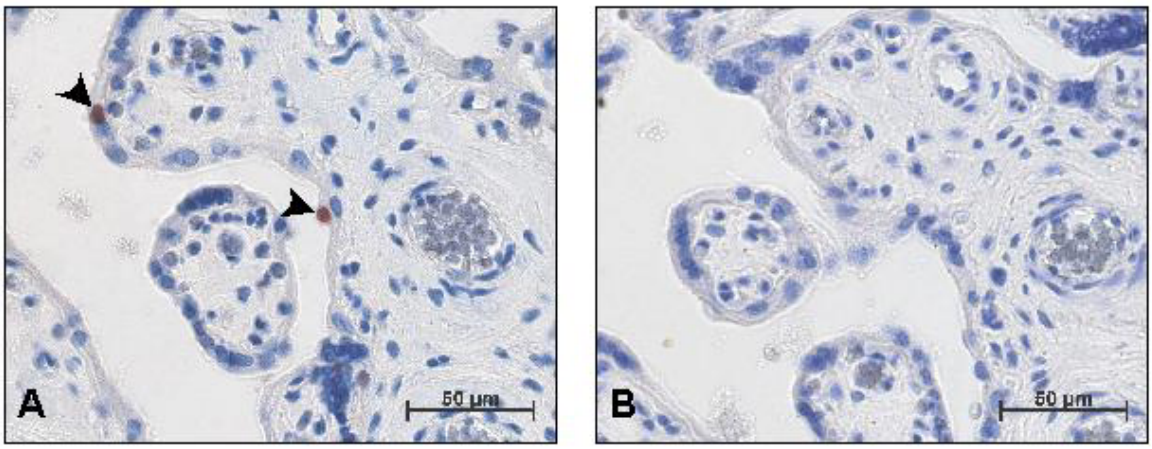


Figure 3.18: Immunolocalisation with antibody p53 (key regulator). P53 was only rarely detected in all study groups and found mainly within specific trophoblasts (A). Panel B shows IgG isotype matched negative controls a parallel section. black arrowhead: syncytiotrophoblast.

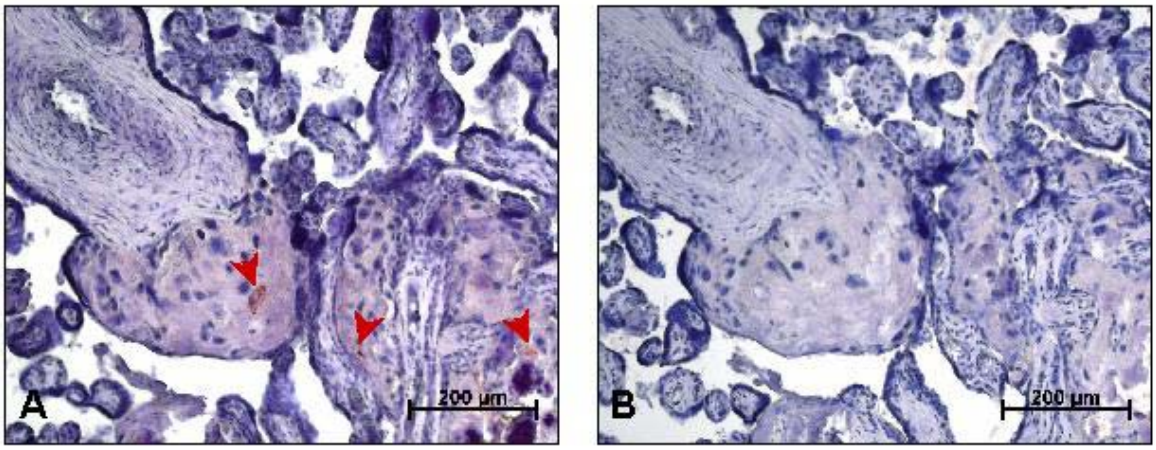


Figure 3.19: Immunolocalisation of cytokeratin 18 neoepitope (clone M30, apoptosis). Cytokeratin 18 neoepitope (A) was rarely labelled in trophoblast cells. Panel B shows IgG isotype matched negative controls a parallel section. red arrowhead: apoptotic extra villous trophoblast cells.

Folate and fatty acid concentrations were correlated with PCNA and p53 expression, the ratio PCNA/p53 and cytokeratin 18 neoepitope. No relationship was found for 5-MTHF supplementation and the expression levels of the proteins. These also did not correlate with the altered proportions of DHA and EPA of the analysed placental lipid fractions. However, EPA within the TG fraction and PCNA levels showed a strong trend for correlation (R^2=0.05, p=0.051) (Figure 3.20).

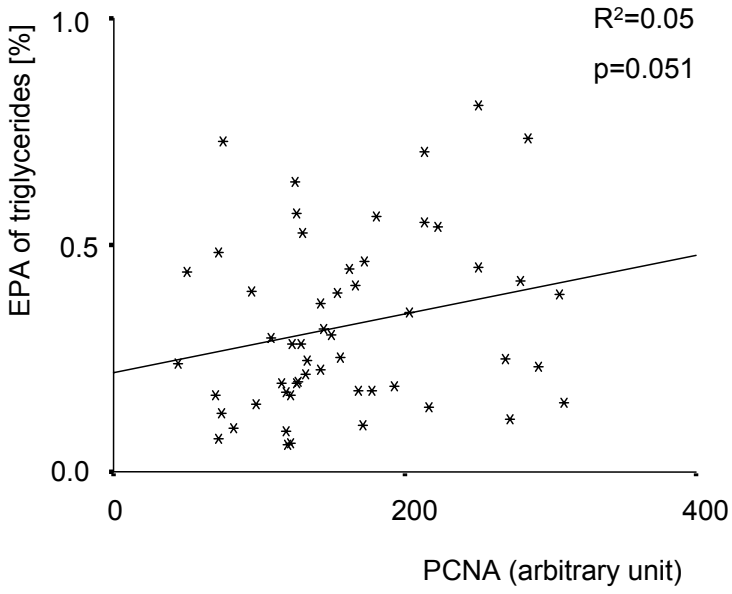


Figure 3.20: Correlation between PCNA expression and EPA content in triglycerides fraction depending on the n-3 LC-PUFA supplementation during the second half of pregnancy.

4 Discussion

4.1 Method development

The described method enabled the analysis of absolute concentrations of fatty acids from placental tissue lipid fractions with a satisfactory precision.

Lakin et al. (1998) determined the relative fatty acid composition of total placental lipids in 10 English women (76), which were similar to our results. This similarity might reflect an almost identical daily intake of fatty acids reported in the Lakin study and in Germany (82). Matorras et al. (2001) described the relative fatty acid composition in placental PL of 78 Spanish women. Differences from these results may be explained by a higher consumption of sea fish and olive oil in Spain compared with Germany (82;83), resulting in higher contents of oleic (13.88% vs. 12.10%), eicosapentaenoic (0.40% vs. 0.11%) and docosahexaenoic acid (5.63% vs. 4.72%), and lower contents of arachidonic acid (22.56% vs. 24.96%) in the Spanish samples. Such diet induced effects have been observed with respect to plasma lipid composition in supplementation studies with fish oil and olive oil (84).

Data on the precision of tissue fatty acid analysis could not be found, but such data have been reported for the analysis of relative fatty acid content in foods. CVs ranging from 0.6% to 10.7% were reported only for those fatty acids with concentrations above 0.1 mg per g fresh sample (85). With the presented method here the quantification limit was lower at only 0.01 mg per g sample. A CV of 10% is sufficient for the detection of clinically relevant differences of the fatty acid content in biological samples in physiological or nutritional studies.

A few papers have reported absolute fatty acid concentrations in plasma or isolated lipoproteins, respectively (86;87). Only one publication has reported absolute fatty acid concentrations in placental tissue (88). Absolute concentrations may be more informative than the percent composition for studying time-dependent processes as well as placental transfer mechanisms. Our method has a good precision for the determination of absolute fatty acid concentrations in PL, TG and NEFA. CE showed a higher variation, possibly because their solubility in methanolic hydrogen chloride is more limited. A higher derivatisation temperature and a longer incubation time might enhance the precision here (78).

The inter-assay shows the same precision as the intra-assay only in the PL fraction. Possibly, the high amount of PL in placental tissue compensated for small alterations

during storage and sample preparation, whereas changes in the NEFA, TG, and CE fractions led to stronger relative variations of the results. CVs of inter-assay for C8-C26 fatty acids in plasma were reported to range from 4.6% to 22.9% (89), compared with the CVs in our study ranging from 4.6% to 30.5%.

With respect to the particular importance of NEFA in the placental fatty acid transfer and metabolism, it is very important to avoid alterations in this fraction as much as possible. PLA2 and triacylglycerol hydrolase catalyse the cleavage of fatty acids from PL and TG, which accumulate in the NEFA fraction (40;90). Within 45 min after delivery the NEFA concentration increased significantly, but within the first 10 min no significant changes occurred (Figure 3.1). We did not observe significant alterations in the quantitatively small TG fraction, which indicates a low activity of triacylglycerol hydrolase. Therefore fatty acids appear to be contributed primarily by hydrolysis from the PL fraction, which is abundant in placental tissue. The proportional composition of total PL and of liberated fatty acids differs because of the preference of PLA2 for the sn-2 position (91) and the asymmetric distribution of acyl chains in PL. Saturated fatty acids are generally esterified at the sn-1 position, while unsaturated fatty acids are preferentially esterified at the sn-2 position (92). This is in agreement with our results where in NEFA arachidonic acid showed the highest proportional increase over time followed by dihomo-γ-linolenic acid and linoleic acid, while the proportions of all saturated fatty acids decreased. We conclude that in order to minimise alterations in the NEFA fraction, placental tissue should be sampled as fast as possible and frozen immediately.

It has been reported that addition of EDTA to the washing solution inhibits PLA2 activity in a concentration dependent manner (93). It was assumed that the penetration of EDTA through an intact plasma membrane is limited, therefore the effect of EDTA in the washing solution to inhibit enzyme activity should be of little benefit, and this was therefore not used.

The distribution of fatty acids in different regions of the placenta is determined by transfer, placental metabolism, and release to the foetal circulation (38). Data on the fatty acid distribution are not available, but differences across the placenta might be assumed. Thus samples from different randomly chosen locations of the placenta were analysed, including central and peripheral locations of maternal and foetal side and the parenchyma. The analysis of the relative fatty acid composition of all lipid fractions indicated no significant differences between the investigated locations. The

absolute concentrations of the PL bound fatty acids showed a homogeneous distribution, in contrast to the fatty acids in NEFA, TG and CE. Therefore for investigations of the relative fatty acid composition in placental lipid fractions, the location of sampling does not need to be exactly defined, while determinations of absolute fatty acid concentrations require explicit definition of the location, usually the centre of the parenchyma.

4.2 Intervention study: placental fatty acid contents

4.2.1 Influence on the placental PL fatty acid profile

4.2.1.1 Comparison of placental PL contents within the placebo group

The main dietary sources for n-3 LC-PUFA are fish and sea food (94). Fatty fish, e.g. salmon, herring, mackerel and anchovy contains the highest amount of DHA and EPA with approximately 1.2 g and 0.8 g per 100 g fish, respectively, whereas white fish like sole, plaice and cod comprises approximately 0.1 g DHA and 0.6 g EPA per 100 g fish (95). In Spain white fish preferentially is consumed, whilst in Germany fatty fish is preferred (95). In Hungary the fish consumption is negligible, which could be related to the availability of food. For example, daily per capita availability of fish in Spain is 75 g, whereas in Hungary only 4 g are available (96). These nutritional habits are reflected in placental PL DHA and EPA contents. The levels of DHA were significantly lower in the Hungarian placentas than in German and Spanish samples and comparable with the placental DHA contents of vegetarians, who exclude meat and fish from their diet (76).

In the study population of Germany and Spain the same contents of n-3 fatty acids in placental PL were found. Matorras et al. reported higher placental DHA contents (5.63±0.86%, mean±S.D.) in a Spanish population recruited in the same area (77) than we have found (4.91±0.19%). However, large variations of daily fish consumption in the Spanish population have been described, which relate not only to geographical region, but also to socio-economical status (97;98).

The maternal intake of n-6 fatty acids seems not to influence the fatty acid pattern in placental PL to the same extent as the n-3 fatty acids. The habitual diet of the Hungarian population contains much more red meat and poultry, the main AA sources (99), compared with the diets in Germany and Spain (96). However, AA contents in placental tissue did not differ significantly between the study populations. This is in

agreement with the observation that only LA content of placental PL differ significantly between vegetarians and omnivores, while AA was very similar (76). This suggests some metabolic regulation of the tissue AA content.

4.2.1.2 Supplementation effect of n-3 LC-PUFA in placental PL fatty acids

After supplementation with n-3 LC-PUFA the DHA contents were similar at approximately 6% in the three national groups. It is unlikely that placental tissue presented an incorporation limit for DHA in membrane PL, because in the retina of five-week-old rats much higher contents up to 42% have been measured (100). The supplementation with daily 500 mg DHA might converge different habitual DHA intake to an average uptake of DHA in the total study population, which is reflected in very similar placental PL contents. This suggested that in all countries the supplementary DHA intake contributed the major portion to the total intake.

It is well documented that the supplementation of DHA and EPA in fish oil enhances the n-3 fatty acid status in plasma in the foetus, which is correlated with beneficial effects for the infant (46;101;102). The group of van Houwelingen supplemented women with fish oil (0.92 g DHA and 1.28 g EPA / d) after week 30 of pregnancy, DHA and EPA concentrations increased in venous umbilical plasma PL in neonates, whereas the arachidonic acid contents decreased (20). Similar results were found in a further cohort study where women were supplemented from week 18 of pregnancy with 1.2 g DHA and 0.8 g EPA per day, (47). The decrease of arachidonic acid in the neonates is not unproblematic, because reduced contents of this n-6 fatty acid may be related to lower growth during the first year of live (103). In our study the supplementation with n-3 LC-PUFA (approximately 500 mg DHA and 150 mg EPA, daily) significantly increased the content of DHA and EPA in membrane PL, whereas in TG and NEFA fractions slight enhancements were measured. However, the supplementation did not alter the arachidonic acid content in the examined placental lipid fractions. This could be related to the high ratio of DHA to EPA by approximately 4:1 in the supplement (104). The supplement used here contained three times more DHA than EPA. Although this caused significant accumulation of n-3 LC-PUFA in placental PL, it did not affect the AA content. AA is of major importance for growth and development of the fetus, and its metabolites influence brain signal transduction in animals (105), thus a limited placental availability of AA might have adverse effects on the neonatal outcome.

4.2.1.3 Influence of the additional 5-MTHF intake on placental PL

Maternal homocysteine concentrations correlated negatively with the DHA contents in foetal erythrocyte PL (53). The presence of 5-MTHF and cobalamine enhanced the re-methylation of homocysteine to methionine (51). Therefore, if maternal 5-MTHF intake improves the foetal DHA status, and the foetal DHA is limited by placental transfer, 5-MTHF might be a factor that improves placental DHA transfer. According to the present data the additional intake of 5-MTHF did not have an influence on the incorporation of the n-3 fatty acids of the placental lipid fractions. Thus, 1) DHA transfer is not influenced or 2) improved DHA transfer is independent of placental DHA content.

4.2.2 Effects of n-3 LC-PUFA intake on placental NEFA, TG and CE fractions

Serum TG and NEFA fatty acid profiles reflect the most recent dietary intake, whereas the fatty acid composition of serum PL and CE change more slowly and reflect the fatty acid compositions of the diet during the preceding weeks. (106). As described above, the dietary fatty acid pattern of the pregnant women was reflected in n-3 LC-PUFA contents in placental PL. However, the placebo groups showed differences of the DHA content in NEFA and TG similar to PL. Although differences between the countries were not statistically significant, DHA content showed the same tendency. The proportions of DHA in NEFA, TG and CE varied more widely (1.9-8.8%, 1.4-17.2% and 1.4-9.3%, respectively) than DHA in PL (3.1-8.1%). This could be related to the much larger PL pool compared with the other lipids fractions. Placental PL seem to reflect the fatty acid profile of the maternal diet over a longer time period, whereas the fatty acid composition of TG and NEFA are more dependent on the most recent diet. The DHA proportion in placental CE seemed to be independent from the recent dietary DHA intake of the mother. The maternal supplementation with n-3 LC-PUFA affected each placenta lipid fraction, which suggested a extensive exchange of fatty acids between the lipid fractions.

The additional n-3 LC-PUFA uptake did not affect the total content of saturated fatty acids, but other n-3 and n-6 LC-PUFA, mainly n-6 DPA. This could be related to the preferential incorporation of saturated fatty acids at the sn-1 position of TG and PL, whereas unsaturated fatty acids are preferentially found at the sn-2 position (92).

Human and animal studies suggested that additional uptake of EPA decreased the AA contents of PL in different tissues, e.g. skeletal muscle, liver and placenta, as well as in maternal and foetal plasma (20;42;107;108). As described above, the low EPA concentration in the supplement did not significantly change the AA content of PL, as well as in NEFA, TG and CE, but a slightly negative correlation between AA and EPA was found in the placental PL and the CE fraction. However, our placental data did not document an effect of the low additional EPA concentration on the AA content in foetal plasma PL. For intervention studies it is of major interest not to change AA contents in placental tissue, considering the importance of this LC-PUFA for foetal growth and development (5).

4.2.3 Distribution of DHA and AA in placental tissue

Differences in the fatty acid composition between lipid fractions resulted from different incorporation rates from the NEFA pool into PL, TG and CE (31). A direct exchange of fatty acids between the esterified placental lipid fractions has not been previously described. Thus, if the NEFA pool was the fatty acid source, its composition should influence the composition of the other fractions. The AA contents correlated between the NEFA and TG fraction, but not between NEFA and PL. This suggested that mechanisms of AA incorporation into TG and PL were different. This was in agreement with a preferential incorporation of AA into the PL fraction, as described in the perfused placenta model (39). Furthermore, in BeWo cells AA was esterified mainly into PL (almost 60%), while less than 35% were found in the TG fraction (41). The latter study also reported that 60% of DHA was incorporated into TG and only 37% were esterified into the PL fraction. This was in agreement with the observations that the DHA content in TG was closely related to the NEFA DHA content.

The selective transfer for individual fatty acids by FABP and fatty acid translocase across the placenta has been suggested as one mechanism to achieve higher LC-PUFA percentages in the foetal than in the maternal circulation (31;38). Mechanisms for a selective release of LC-PUFA to the foetal circulation are not known. In placental tissue AA and DHA were highly enriched in all lipid fractions relative to maternal and foetal plasma lipids (15). Take together, this suggested a preferential uptake rather than a preferential release, as mechanism for the selective LC-PUFA transfer across the placenta. Selectivity for the accumulation of AA and DHA might be impor-

tant functions of placental tissue, to maintain a constant supply of the fetus with n-3 and n-6 LC-PUFA.

4.3 Intervention study: Western blot and Immunohistochemistry

One of the important questions of this study was the potential effect of supplementation with n-3 LC-PUFA (DHA and EPA) and/or folic acid on proliferation and apoptosis of different cells in the human placenta. To address this, key proteins were selected that reflect the extent of both processes as well as their regulators. PCNA is a well-known cell cycle marker. In different studies PCNA has been shown to be a useful tool in evaluating the proportion of proliferating cells (109) and it is immunolocalised mainly in the nuclei of cytotrophoblast cells (57). Although the long half-life of about 20 hours may lead to absolute overestimations (57), this is unlikely to affect group comparisons. Interestingly, PCNA levels were only high in the mixed n-3 LC-PUFA and 5-MTHF group, whereas the treatment with either n-3 LC-PUFA or 5-MTHF had no effect. P53 levels were not changed in the combination group, thus it is unclear at present what might have caused the increase in PCNA levels in these group. Particularly, p53 is a master switch regulator in the cell and plays an important role as a cell cycle checkpoint protein, and in invasion and apoptosis. The protein is localised mainly in nuclei of extravillous trophoblasts, in low proportions also in cytotrophoblast and syncytiotrophoblast cells (110). The ratio of PCNA protein and p53 decided the function of PCNA. If the ratio was low, DNA repair took place and if the ratio was high, DNA replications occurred (111). However, a high abundant appearance of PCNA in the cell induces proliferation (111). Apoptotic cells were detected using an antibody for cytokeratin 18 neoepitope, this neoepitope is only formed by caspase activity when cells undergo apoptosis and immunolocalised by M30 antibody in cyto- and syncytiotrophoblast cells as well as in extravillous trophoblast cells (112;113). The detection of cytokeratin 18 neoepitope now is more reliable to identify apoptosis then the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling) method in placental tissue (112-114). In addition, this antibody for cytokeratin 18 neoepitope can be used in both immunohistochemistry as well as in western blot (114), which was a criteria when choosing the method.

It was shown that a high intake of fish oil, especially DHA, during pregnancy led to an elongation of pregnancy and an increase of birth weight (45). Therefore, we expected

that the proliferation rate in the placenta at the end of pregnancy must be enhanced. Our results demonstrated increased proliferation rates in women who have had an additional uptake of n-3 LC-PUFA and folic acid in combination, but not in women who supplemented either n-3 LC-PUFA or 5-MTHF. The enhanced proliferation was only related to trophoblastic cells, which was reflected in the total proportion, whereas endothel and other stromal cells were not affected. These findings are notable, because in fish oil supplementation studies enhanced DHA contents were measured in the foetal circulation (20). Thus, if the effect would depend on the additional intake of DHA, increased proliferation rates should also exist in endothel cells. Complementary, enhanced PCNA contents were found in third trimester trophoblasts of women, who smoked during gestation (115). It is unlikely that smoking have caused the changes seen here, because the distribution of the smokers in the four groups was not different (X^2 = 0.680). The data suggested that the combination of folic acid and DHA plays an important role in this processes, but mechanism remained unclear.

On the other hand, EPA inhibited cell proliferation while DHA increased apoptosis (116;117) and the influence of DHA and EPA on apoptosis seems to be mediated by different mechanism. While DHA effects on apoptosis are attributed mainly to its incorporation into the PL bilayer, EPA apoptotic effects are associated to TG of lipid bodies within the cell. Furthermore, controversially effects of DHA on the apoptosis rate are described in different tissues. Dietary supplementation increased apoptotic cell death in colonic and hepatocarcinoma cells (116;118), whereas other studies demonstrated inhibitory effects on apoptosis in neuronal and lymphoma cells (119;120). In the present study the higher DHA contents in membrane PL as well as the slightly enhanced DHA proportions in cytosolic TG and NEFA, had no effect on apoptosis rates in full term placentas.

The uptake of high concentrations of DHA or low ratios of DHA to EPA (~1:1) decreased the concentration of arachidonic acid in plasma PL and TG (20;42;104). It was demonstrated that an increase of arachidonic acid in NEFA or altered arachidonic acid pools in individual membrane phospholipids induce apoptosis in different cell types (121). Here we found no effect of DHA and EPA on arachidonic acid contents in placental lipid fractions analysed. The absence of such changes may explain the lack of effect on proliferation and apoptosis.

In vitro studies indicated that folate deficiency induces apoptosis in human trophoblastic cells, but there are no data available how folate supplementation *in vivo*

affects placental apoptosis and proliferation (122). The supplementation of folic acid did not alter apoptosis, reflecting well-known differences between the conditions *in vivo* and *in vitro*.

4.4 Conclusion

The evaluated method for placental tissue fatty acid analysis presented here shows a reasonable precision for the analysis of fatty acids in different lipid fraction of placental tissue. The good reproducibility of the absolute fatty acid concentrations offers opportunities for further investigations of time-dependent processes, placental transfer functions and for the study of different nutritional and physiological factors.

The additional intake of n-3 LC-PUFA enhanced the DHA and EPA contents in placental PL, NEFA, TG and CE, with no adverse effect on the AA content. Considering the importance of AA and DHA for foetal growth and development, women with a low intake of n-3 fatty acids could increase their DHA status in placenta with an additional intake of 500 mg DHA per day during the second half of pregnancy.

Details of the placental transfer mechanisms remain to be elucidated, but it could be shown that fatty acids from the maternal circulation are reflected in placental PL, TG and NEFA fractions, and placental uptake seems to be more selective than release. The PL fraction (or total placental lipids) is best used for studying maternal dietary background. The supplementation with 5-MTHF did not affect the PL fatty acid pattern in placenta.

The effect of an additional intake of n-3 LC-PUFA and folic acid on placental apoptosis and proliferation remained unclear. No changes of proliferation were found if pregnant women were supplemented either with n-3 LC-PUFA or folic acid. Combined administered n-3 LC-PUFA and 5-MTHF enhanced the proliferation rate in trophoblast cells, but not in endothel cells or other stromal cells. In contrast, the apoptosis rates were not affected in all treatment groups, which indicated for the combined supplemented group a changed homeostasis.

5 Summary

A controlled double-blind randomised clinical trial was accomplished, which compared the effects of dietary supplementation with docosahexaenoic acid (DHA) and/or 5 methyl-tetrahydrofolate (5-MTHF) from week 20 of gestation until child birth in mothers from different countries and corresponding dietary differences. Women from Spain (n=100, high intake of sea fish, major DHA source), Germany (n=48, medium intake) and Hungary (n=54, low intake) were enrolled in the study. The offered dietary supplement contained micronutrients according to European recommendations, but differed in their DHA and folate contents: a) 500 mg DHA per day, b) 400 µg 5-MTHF / d c) 500 mg DHA + 400 µg 5-MTHF / d) control (0 g DHA, 0 mg 5-MTHF). Placental tissue was collected at child birth and the fatty acid profiles of different lipid fractions, e.g. phospholipids (PL), nonesterified fatty acids (NEFA), triglycerides (TG), and cholesterolesters (CE), were analysed by gas chromatography. Furthermore, in placentas of a Spanish subgroup, the apoptosis- and proliferation rates were determined using the antibodies against PCNA (proliferation cell nucleus antigen), p53 (tumor suppressor gene) and cytokeratin 18 neoepitope (apoptosis marker).

5.1 Method development for fatty acid analysis in placental tissue

A critical point was to develop and evaluate a method for the determination of fatty acid concentrations in placental tissue. Lipids were extracted from placental tissue with a chloroform methanol mixture and PL, NEFA, TG, and CE were isolated by thin layer chromatography. Individual lipid fractions were derivatised with methanolic hydrochloric acid and the fatty acid methyl esters were quantified by gas chromatography with flame ionisation detection.

- The coefficient of variation (CV of intra-assay, n=8) of absolute concentrations were evaluated for fatty acids showing a tissue content > 0.01 mg/g. CV ranged from 4.6% – 11.0% for PL, 6.4% – 9.3% for NEFA, 6.1% - 8.9% for TG, and 11.4% - 16.3% for CE.
- The relative fatty acid composition across a term placenta indicated no differences between samples of central and peripheral locations of maternal and foetal

site (CVs 0.5% – 9.9%), whereas the absolute fatty acid concentrations were only reproducible in the PL fraction (CVs 7.0% – 12.8%).

The method showed a reasonably high precision that was well suitable for physiological and nutritional studies.

5.2 Differences of the placental PL fatty acid profile between the centres

It was hypothesised that differences in habitual diets in different parts of Europe influence the fatty acid content of placental phospholipids.

- In the placebo groups, DHA proportions were similar in Germany and Spain (5.04±0.28% vs. 4.91±0.19%, respectively), but lower in Hungary (3.95±0.18%, $p<0.05$). The similarity between Germany and Spain could be related to large variations of daily fish consumption in the Spanish population, which not only depend on geographical region, but also to socio-economical status.
- Arachidonic acid (AA) contents in placental tissue did not differ significantly between the study populations, which is in agreement with some metabolic regulation of the tissue AA content.

Thus, the maternal intake of n-6 fatty acids seems not to influence the fatty acid pattern in placental PL to the same extent as the n-3 fatty acids.

5.3 Effect of the supplementation on the placental fatty acid profile

It was hypothesised that the supplementation with n-3 fatty acids changes the fatty acid profile in placental lipid fractions.

- In the total study population DHA contents were higher in placental PL, NEFA, TG and CE after n-3- LC-PUFA supplementation compared with the non-fish oil supplemented group (5.88%±0.11 vs. 4.77%±0.10, 5.28%±0.15 vs. 4.12%±0.13, 6.80%±0.33 vs. 4.64%±0.22, and 3.98%±0.14 vs. 3.00%±0.10, respectively, $p<0.05$).
- Although the enhancement of n-3 LC-PUFA was shown in each placental lipid fraction, the PL fraction (or total placental lipids) are best used for studying maternal dietary background.

- In placental tissue AA and DHA were highly accumulated in all lipid fractions relative to maternal and foetal plasma lipids.

The results suggest that the placental uptake is more selective than release.

5.4 Effect of the supplementation on the placental arachidonic acid content

- AA percentages in placental phospholipids (Placebo: 22.2%±0.3; DHA: 22.3%±0.3; 5-MTHF: 22.1%±0.2; Combined: 21.6%±0.3) were not significantly different between groups, whereas the EPA contents in the "fish-oil" groups (0.17%±0.01, 0.20%±0.02, DHA and combined, respectively) were higher ($P<0.05$) than in the "non-fish oil" groups (0.11%±0.01, 0.12%±0.01, Placebo and 5-MTHF, respectively).
- Negative correlations between the fatty acids were observed in phospholipids ($r=-0.263$) and cholesterolesters ($r=-0.262$) but not in the other fractions.
- In phospholipids an EPA increase of 0.1% was associated with a lowering of AA content by approximately 0.5%.

N-3 fatty acid supplementation up to the tested dose seems not to interfere with AA dependent processes in the placenta and might be recommended.

5.5 Determination of apoptosis and proliferation in placental tissue

The effect of an additional intake of n-3 LC-PUFA and 5-MTHF on placental apoptosis and proliferation remained unclear. No changes of proliferation were found if pregnant women were supplemented either with n-3 LC-PUFA or 5-MTHF. Combined administered n-3 LC-PUFA and 5-MTHF enhanced the proliferation rate in trophoblast cells (combined group 12.8%, placebo group 7.3%), but not in endothel cells or other stromal cells. In contrast, the apoptosis rates were not affected in all treatment groups, which indicated for the combined supplemented group a changed ratio between apoptosis and cell proliferation.

6 Zusammenfassung

In einer in Spanien, Ungarn und Deutschland durchgeführten Interventionsstudie wurden Frauen während der zweiten Schwangerschaftshälfte mit Docosahexaensäure (DHA) und/oder 5-Methyltetrahydrofolsäure (5-MTHF) supplementiert. Die zusätzlich zur täglichen Nahrung verabreichten Präparate enthielten jeweils die den europäischen Empfehlungen entsprechenden Mikronährstoffe. Der Gehalt an DHA und 5-MTHF war gruppenspezifisch unterschiedlich: 1) 500 mg DHA, 2) 400 µg 5-MTHF, 3) 500 mg DHA + 400 µg 5-MTHF, 4) Placebo. Die Zuteilung der Schwangeren zu einer der vier Gruppen erfolgte randomisiert und doppelblind.
Im Rahmen dieser Arbeit wurde in der Plazenta das Fettsäurenprofil in Phospholipiden (PL), nicht veresterten Fettsäuren (NEFA), Triglyzeriden (TG) und Cholesterolestern (CE) mittels Gaschromatographie analysiert. Darüber hinaus erfolgte bei einem Teil der spanischen Plazenten die Bestimmung der Apoptose- und Proliferationsrate mittels Western Blot und Immunhistologie.

6.1 Methodenentwicklung für die Bestimmung von Fettsäuren in Gewebe

Die Entnahme des Probenmaterials aus der Plazenta erfolgte innerhalb von 10 Minuten nach der Geburt (Zentrum, 0,3 g/Probe). Mittels Chloroform-Methanol-Gemisch (2:1 v/v + 5g/l Buthylhydroxytoluol) wurden die Lipide aus dem Gewebe extrahiert und durch Dünnschichtchromatographie in Phospholipide (PL), NEFA, TG und CE aufgetrennt. Die sich anschließende Umesterung der Lipidfraktionen zu leicht flüchtigen Fettsäuremethylestern (FAME) erfolgte säurekatalysiert in 3 M methanolischer HCl (45 min, 85°C). FAME mit einer Kettenlänge von 14-24 Kohlenstoffen wurden mittels Gaschromatographie quantifiziert.

- Der Variationskoeffizient (VK) des Intra-Assays von Fettsäuren mit einer Konzentration größer als 0,01 mg pro g Gewebe betrug in PL 4,6% - 11,0%, NEFA 6,4% - 9,3%, TG 6,1% - 8,9% und CE 11,4% - 16,3% (n=8).
- Die prozentuale Zusammensetzung des Fettsäurenmuster über die Plazenta (Zentrum, Randbereiche, mütterliche und foetale Seite, n=12) war in den untersuchten Lipidfraktionen einheitlich (VK 0,5% - 9,9%), während die einzelnen Fettsäurekonzentrationen in NEFA (VK 8,0% - 13,4%), TG (6,1% - 27,9%) und CE

(10,7% - 42,1%) stark variierten und nur in den PL eine ausreichende Reproduzierbarkeit erzielten (VK 5,7% - 12,8%).

- Der Vergleich von Fettsäurekonzentration in NEFA, TG und CE zwischen verschiedenen Plazenten erfordert eine definierte Stelle der Probenentnahme. Für die Bestimmung der Fettsäuren in PL oder in Gesamtlipiden spielt die Entnahmestelle keine Rolle. Die Methode zur Fettsäurebestimmung in plazentarem Gewebe erzielte in allen untersuchten Lipidfraktionen eine ausreichend hohe Präzision und ist für physiologische und ernährungsbezogene Studien anwendbar.

6.2 Unterschiedliche Fettsäuremuster der Plazenten in Deutschland, Spanien und Ungarn

Es wurde vermutet, dass die unterschiedlichen Ernährungsgewohnheiten der einzelnen Länder das Fettsäuremuster der Plazenta Phospholipide beeinflusst.

- In der mit Placebo behandelten Gruppe war der DHA Gehalt in den Plazenten der ungarischen Mütter wesentlich niedriger (3,95%±0,18, $p<0,05$) als der der deutschen und spanischen Mütter (5,04%±0,28% vs. 4,91±0,19%). Dies stand im Gegensatz zur ursprünglichen Überlegung, dass die DHA-Aufnahme über die Nahrung in Spanien gegenüber Deutschland erhöht ist. Die Beobachtungen dieser Studie sind vermutlich auf die in Spanien gewöhnlich großen regionalen Unterschiede im Fischkonsum als auch auf den sozio-ökonomischen Status zurückzuführen.
- Der prozentuale Anteil der Arachidonsäure (AA) in den Plazenta Phospholipiden unterschied sich im Mittel nicht zwischen den einzelnen Zentren.
- Die Aufnahme von langkettigen n-6 Fettsäuren mit der mütterlichen Nahrung beeinflusst das Fettsäurenprofil der Plazenta nicht im gleichen Umfang wie das der langkettigen n-3 Fettsäuren.

6.3 Einfluss der Supplementation auf das Fettsäurenprofil der Plazenta

Es wurde überprüft, welchen Einfluss die Supplementation auf die Fettsäurenmuster der einzelnen Lipidfraktionen hat. Dafür sind die Mütter, die das modifizierte Fischöl einnahmen, zu einer Gruppe zusammengefasst worden. Die Kontrollgruppe bekam die Folsäure und Placebo Präparate.

- Nach der Intervention stieg der DHA Gehalt der mit n-3 Fettsäuren supplementierten Gruppe gegenüber der Kontrollgruppe an (PL 5.88%±0.11 vs. 4.77%±0.10, NEFA 5.28%±0.15 vs. 4.12%±0.13, TG 6.80%±0.33 vs. 4.64%±0.22, and CE 3.98%±0.14 vs. 3.00%±0.10, $p<0.05$).
- Für die Untersuchung nahrungsbedingter Einflüsse auf das Fettsäuremuster der Plazenta eignen sich Phospholipide (bzw. der Gesamtlipidgehalt) aufgrund des hohen Anteils am Gesamtlipidgehalt am besten.
- AA und DHA werden gegenüber dem mütterlichen und dem foetal Kreislauf stark angereichert, was in Bezug auf den Transfer eher auf eine selektive Aufnahme als auf eine selektive Abgabe an bestimmten Fettsäuren schließen lässt.

6.4 Der Einfluss der Supplementierung auf den Arachidonsäure Gehalt in der Plazenta

- Die prozentualen Anteile der Arachidonsäure in den Phospholipiden zwischen den Gruppen (Placebo: 22,2%±0,3; n-3 LC-PUFA: 22,3%±0,3; 5-MTHF: 22,1%±0,2; Kombinierte Gruppe: 21,6%±0,3; mean±SEM) waren nicht signifikant unterschiedlich, während die Eicosapentaensäure Gehalte der kombinierten Gruppe (0,20%±0,02) und der DHA Gruppe (0,17%±0,01) höher ($P<0,05$) als die der 5-MTHF Gruppe (0,12%±0,01) und Placebo Gruppe (0,11%±0,01) waren.
- Eine negative Korrelation zwischen Arachidonsäure und Eicosapentaensäure wurde in den Phospholipiden ($r= -0,263$) und den Cholesterolestern ($r= -0,262$) berechnet, während die beiden Fettsäuren in den anderen Lipidfraktionen nicht miteinander korrelierten.
- Eine Erhöhung der Eicosapentaensäure um 0,1 % in den Phospholipiden erniedrigte den Arachidonsäure Gehalt um etwa 0,5%.
- Die Studie zeigt, dass während der Schwangerschaft eine tägliche Aufnahme von etwa 150 mg EPA zwischen den Gruppen keinen Unterschied im AA Gehalt in den Phospholipiden der Plazenta bewirkt, obwohl eine negative Korrelation zwischen den Anteilen der beiden Fettsäuren aufgezeigt werden konnte. Der Einfluss der verabreichten EPA Dosis auf den AA Gehalt, und damit auf AA abhängige Prozesse, scheint klein zu sein

6.5 Bestimmung der Apoptose und Proliferationsrate in Plazentagewebe

- Die Proliferationsrate der Trophoblasten wird durch die kombinierte Gabe von n-3 Fettsäuren und 5-MTHF gesteigert (12,8% vs. 7,3%), während Endothelzellen und Stromazellen nicht beeinflußt waren.
- Es konnte keine veränderte Apoptoserate nachgewiesen werden
- Einflüsse auf Apoptose- und Proliferationsvorgänge in der Plazenta durch die zusätzliche DHA und Folsäure Einnahme während der Schwangerschaft bleiben zum jetzigen Zeitpunkt ungeklärt.

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8 Index of Tables and Figures

9 Attachment

9.1 Figures

Figure 9.1: Western blot: flow sheet of the membrane treatment

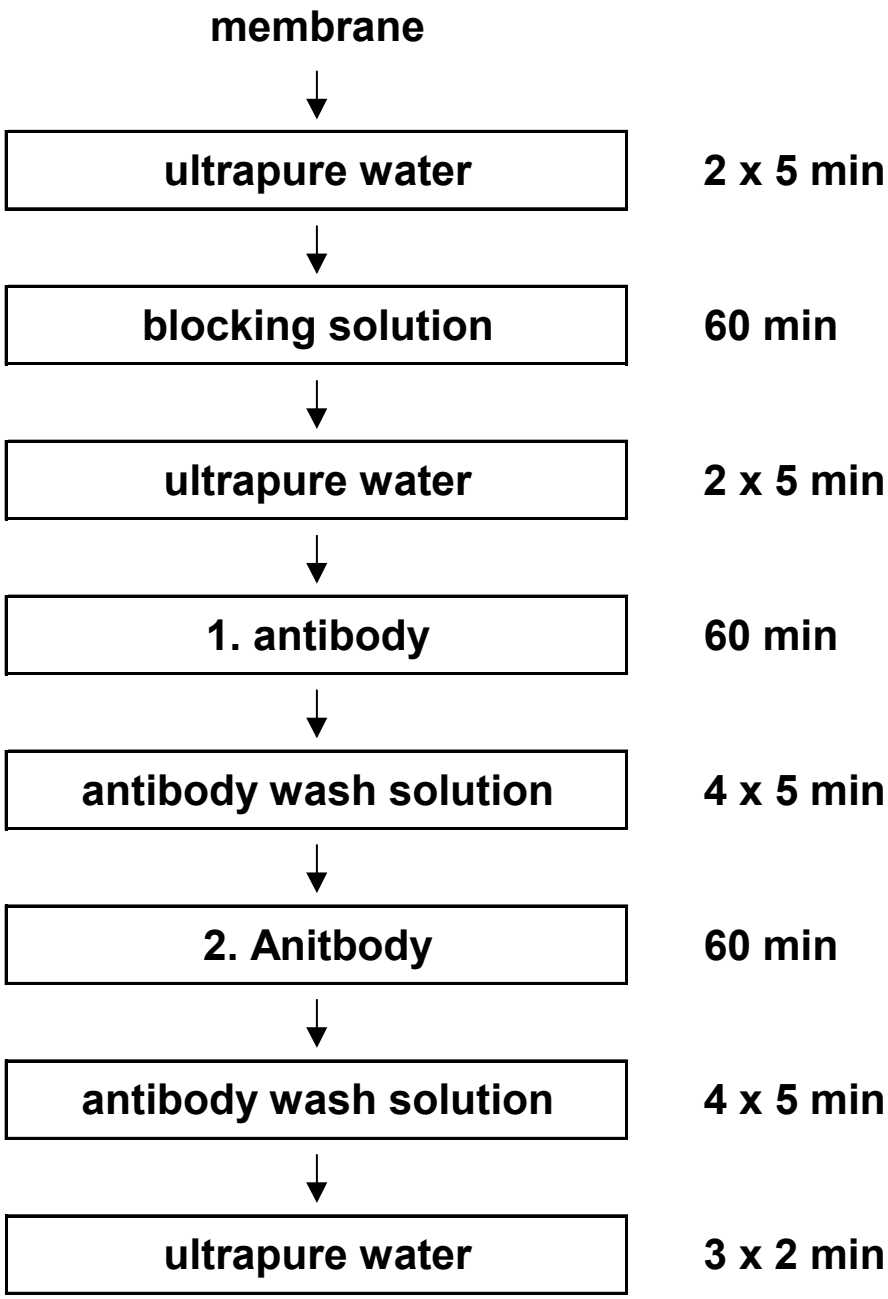
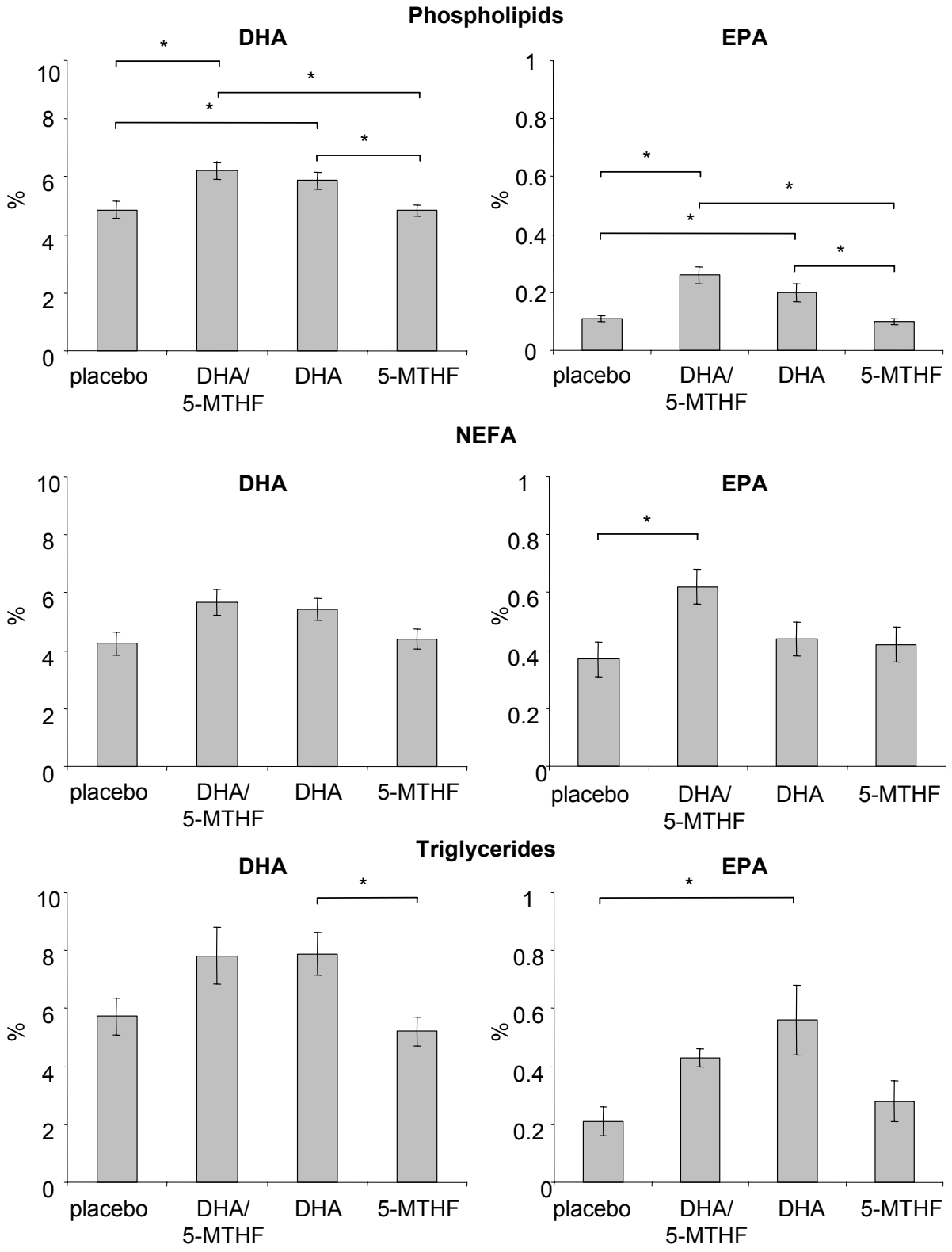


Figure 9.2: Flow sheet for immunohistochemistry

xylene	2 x 10 min
xylene/ethyl alcohol (1:1 by vol.)	10 sec
ethyl alcohol 100% by vol.	10 sec.
ethyl alcohol 96% by vol.	10 sec
ethyl alcohol 70% by vol.	10 sec.
ultrapure water	10 sec
ultrapure water	10 sec
autoclave / microwave	3 min
TBS	5 min
block with activated human serum (20%)	10 min
Antibody	60 min
TBS + 0.05% Tween	3 x 2 min
biotinylated goat anti mouse antibody	15 min
TBS + 0.05% Tween	3 x 2 min
streptavidin-HRP	10 min
TBS + 0.05% Tween	3 x 2 min
AEC staining	< 5 min
ultrapure water	2 x 2min
Haemalumn counterstaining	< 5 sec
Ammonium water (1%)	10 sec
ultrapure water	2 x 2 min

Figure 9.3: Proportions of DHA and EPA (% w/w) in placental tissue PL, TG and NEFA. The groups were supplemented either with placebo (n=12), n-3 LC-PUFA / 5-MTHF (n=11), n-3 LC-PUFA (n=16) or 5-MTHF (n=15). (*p<0.05).



9.2 Tables

Table 9.1: Fatty acid content of the placental NEFA pool 4.5 min (t1) vs. 44.5 min (t2) after delivery (% w/w). Paired t-test was used to determine differences between the time points (n=4, *p<0.05).

	t1	t2
	mean ± SD	mean ± SD
Saturated fatty acids		
C14:0	0.89 ± 0.12	0.79 ± 0.17
C16:0	40.92 ± 5.96	36.62 ± 3.61
C18:0	17.23 ± 2.43	16.34 ± 1.83
C20:0	0.81 ± 0.28	0.64 ± 0.06
C22:0	2.98 ± 0.63	2.13 ± 0.21
C24:0	2.02 ± 0.52	1.53 ± 0.37*
Monounsaturated fatty acids		
C16:1n-7	0.76 ± 0.26	0.78 ± 0.21
C18:1n-9	8.51 ± 2.03	9.25 ± 1.50
C18:1n-7	1.11 ± 0.30	1.22 ± 0.24*
C20:1n-9	0.18 ± 0.08	0.23 ± 0.05
C24:1n-9	0.59 ± 0.19	0.54 ± 0.15
Trans fatty acids		
C18:1 t	0.20 ± 0.10	0.23 ± 0.06
n-9 PUFA		
C20:3n-9	0.13 ± 0.06	0.17 ± 0.06
n-6 PUFA		
C18:2n-6	6.05 ± 0.80	7.16 ± 0.38*
C18:3n-6	0.74 ± 0.82	0.58 ± 0.56
C20:3n-6	2.77 ± 0.88	3.74 ± 1.03
C20:4-n6	9.91 ± 3.29	12.75 ± 2.05
C22:4n-6	0.66 ± 0.29	0.80 ± 0.09
C22:5n-6	0.48 ± 0.16	0.58 ± 0.07
n-3 PUFA		
C18:3n-3	0.12 ± 0.05	0.14 ± 0.03
C20:5n-3	0.17 ± 0.08	0.27 ± 0.09
C22:5n-3	0.30 ± 0.21	0.43 ± 0.13
C22:6n-3	1.94 ± 0.80	2.58 ± 0.82

Table 9.2: Variations of selected fatty acids within different placenta locations in two placentas (CV in %)

Placenta 1 (n=12):	coefficient of variation of the fatty acid concentration				coefficient of variation of the fatty acid composition			
fatty acid	PL	NEFA	TG	CE	PL	NEFA	TG	CE
C16:0	7,0	12,2	25,0	31,5	0,5	3,9	5,9	6,6
C18:0	7,1	10,0	17,3	**31,0**	2,7	3,4	6,9	**14,1**
C18:1n-9	7,1	10,3	25,1	37,7	1,7	4,3	5,3	1,8
C18:2n-6	7,2	10,4	27,9	42,1	1,8	4,0	7,6	7,5
C18:3n-6	**26,6**	n.d	n.d	n.d	**23,5**	n.d	n.d	n.d
C20:4n-6	7,4	13,4	21,1	35,8	1,3	4,5	5,8	8,7
C22:5n-6	8,3	**8,3**	**12,8**	**31,7**	4,9	**6,1**	**18,9**	**19,6**
C18:3n-3	**15,4**	n.d	n.d	n.d	**15,7**	n.d	n.d	n.d
C20:5n-3	**16,0**	n.d	n.d	n.d	**11,2**	n.d	n.d	n.d
C22:5n-3	9,3	**14,5**	**20,9**	n.d	5,0	**10,1**	**12,7**	n.d
C22:6n-3	12,8	**11,6**	**15,9**	**40,4**	9,1	**11,7**	**20,8**	**9,1**

Placenta 2 (n=11):	coefficient of variation of the fatty acid concentration				coefficient of variation of the fatty acid composition			
fatty acid	PL	NEFA	TG	CE	PL	NEFA	TG	CE
C16:0	5,7	8,0	6,3	11,4	1,6	2,7	4,0	8,7
C18:0	5,6	8,2	10,4	**23,6**	1,9	3,5	4,3	**17,2**
C18:1n-9	5,9	11,3	6,1	13,4	3,4	4,9	5,8	9,3
C18:2n-6	6,9	12,9	7,7	10,7	1,8	5,5	2,8	9,9
C18:3n-6	**11,0**	n.d	n.d	n.d	**11,1**	n.d	n.d	n.d
C20:4n-6	9,0	13,0	12,7	14,0	3,2	4,7	7,4	8,5
C22:5n-6	6,3	**17,7**	**11,4**	**23,6**	5,8	**10,0**	**8,3**	**19,1**
C18:3n-3	**15,1**	n.d	n.d	n.d	**10,4**	n.d	n.d	n.d
C20:5n-3	**14,3**	n.d	n.d	n.d	**10,1**	n.d	n.d	n.d
C22:5n-3	8,6	**16,0**	**20,1**	n.d	5,1	**9,6**	**14,9**	n.d
C22:6n-3	9,0	**16,2**	**18,2**	**17,5**	5,6	**8,5**	**14,4**	**14,2**

Bold data show the coefficient of variation of concentrations < 0.010 mg/g. n.d: concentrations < 0.001 mg/g not detected

PL: phospholipids, NEFA: non-esterified fatty acids, TG: triglycerides, CE: cholesterol esters

* The total fatty acid content of one sample differed more than four standard deviations from the mean and therefore was considered an outlier and excluded from the results.

Table 9.3: Intra-Assay (n=8) Reproducibility of Fatty Acid Concentrations of Placental Phospholipids, Non-esterified Fatty Acids, Triglycerides and Cholesterolesters (mg/g)

fatty acids	PL		NEFA		TG		CE	
	mean	CV	mean	CV	mean	CV	mean	CV
Saturated fatty acids								
C14:0	0.031	6.9	0.003	10.6	0.004	5.6	0.004	19.2
C16:0	1.728	5.3	0.102	7.5	0.075	6.5	0.038	15.5
C18:0	0.771	5.3	0.064	6.4	0.031	7.4	0.008	29.7
C20:0	0.023	9.1	0.002	16.3	n.d		n.d	
C22:0	0.089	4.9	0.003	10.2	n.d		n.d	
C24:0	0.121	5.8	0.004	13.8	n.d		n.d	
Monounsaturated fatty acids*								
C16:1n-7	0.028	4.9	0.004	6.4	0.004	7.0	0.007	8.8
C18:1n-7	0.100	5.0	0.007	7.2	0.005	8.4	0.004	21.7
C18:1n-9	0.523	4.8	0.051	6.5	0.045	8.5	0.042	16.3
C20:1n-9	0.015	6.3	0.001	8.1	n.d		n.d	
C24:1n-9	0.089	7.1	0.003	12.1	n.d		n.d	
n-9 PUFA								
C20:3n-9	0.013	11.0	0.001	9.4	n.d		n.d	
n-6 PUFA								
C18:2n-6	0.500	4.6	0.033	6.4	0.026	6.7	0.078	11.4
C18:3n-6	0.005	16.9	0.001	9.8	0.001	7.3	0.002	8.9
C20:3n-6	0.309	5.9	0.019	9.3	0.017	6.1	0.006	25.6
C20:4n-6	1.591	5.9	0.088	8.0	0.042	8.9	0.023	12.4
C22:5n-6	0.062	5.4	0.004	9.5	0.003	10.5	0.001	14.9
n-3 PUFA								
C18:3n-3	0.003	14.4	0.001	9.3	0.001	7.8	0.001	16.6
C20:5n-3	0.007	6.3	0.002	7.6	0.001	8.4	0.001	16.2
C22:5n-3	0.053	5.8	0.003	9.7	0.003	7.3	n.d	
C22:6n-3	0.301	7.4	0.017	13.9	0.012	7.0	0.005	21.9

*Erucic acid (C22:1n-9) content is not reported because of the coelution with an unidentified peak.
n.d: concentrations < 0.001 mg/g not determined

Table 9.4: Intra-Assay (n=8) Reproducibility of the Fatty Acid Composition of Placental Phospholipids Non-esterified Fatty Acids, Triglycerides and Cholesterolesters (% w/w)

fatty acids	PL		NEFA		TG		CE	
	mean	CV	mean	CV	mean	CV	mean	CV
Saturated fatty acids								
C14:0	0.48	7.0	0.77	8.0	1.47	8.2	1.71	8.8
C16:0	27.10	0.4	24.67	2.3	26.97	1.8	17.02	2.0
C18:0	12.10	2.4	15.45	3.1	11.28	3.0	3.58	17.6
C20:0	0.35	10.5	0.40	14.9	n.d		n.d	
C22:0	1.39	2.7	0.81	7.0	n.d		n.d	
C24:0	1.90	4.1	0.91	9.3	n.d		n.d	
Monounsaturated fatty acids*								
C16:1n-7	0.43	4.8	0.86	3.4	1.34	3.6	3.34	7.5
C18:1n-7	1.58	1.1	1.59	1.2	1.93	2.4	1.65	8.9
C18:1n-9	8.21	2.3	12.30	2.2	16.26	3.0	18.87	2.7
C20:1n-9	0.24	6.4	0.34	3.2	n.d		n.d	
C24:1n-9	1.39	7.0	0.66	7.7	n.d		n.d	
n-9 PUFA								
C20:3n-9	0.20	8.8	0.29	10.4	n.d		n.d	
n-6 PUFA								
C18:2n-6	7.84	1.9	8.00	2.2	9.30	5.3	35.23	5.1
C18:3n-6	0.07	14.2	0.10	7.4	0.20	7.5	0.69	6.4
C20:3n-6	4.85	2.1	4.64	4.9	6.18	4.9	2.60	13.3
C20:4n-6	24.96	2.7	21.32	2.3	15.22	4.5	10.56	4.5
C22:5n-6	0.98	4.3	0.92	5.9	1.22	6.5	0.64	9.1
n-3 PUFA								
C18:3n-3	0.04	13.2	0.22	11.7	0.30	7.7	0.54	3.7
C20:5n-3	0.11	8.3	0.41	6.5	0.27	4.5	0.45	4.7
C22:5n-3	0.83	3.3	0.77	4.8	1.02	4.2	n.d	
C22:6n-3	4.72	5.5	3.99	10.7	4.50	9.0	2.08	11.3

*Erucic acid (C22:1n-9) content is not reported because of the coelution with an unidentified peak.
n.d: concentrations < 0.001 mg/g not determined

Table 9.5: Inter-Assay (n=8) of the Fatty Acid Content in Placental Tissue Expressed as Coefficient of Variation (CV in %)

	CV of the absolute fatty acid concentration				CV of the relative fatty acid composition			
fatty acid	PL	NEFA	TG	CE	PL	NEFA	TG	CE
saturated fatty acids								
C14:0	7.5	**10.6**	**14.3**	**16.4**	10.9	**20.4**	**20.4**	**12.8**
C16:0	4.6	17.4	13.7	8.0	1.8	6.3	12.4	3.6
C18:0	5.4	18.7	22.4	**12.9**	1.7	4.4	6.5	**14.0**
C20:0	13.5	**10.7**	n.d	n.d	14.3	**14.4**	n.d	n.d
C22:0	7.6	**14.1**	n.d	n.d	7.2	**16.3**	n.d	n.d
C24:0	8.2	**26.1**	n.d	n.d	11.6	**20.4**	n.d	n.d
Monounsaturated fatty acids								
C16:1n-7	6.5	**36.3**	**18.0**	**16.9**	8.5	**20.6**	**31.9**	**14.3**
C18:1n-7	6.2	**31.0**	**16.9**	**14.1**	5.3	**10.4**	**11.2**	**10.4**
C18:1n-9	5.5	26.6	16.8	13.8	4.4	5.9	5.4	4.4
C20:1n-9	5.2	**27.7**	n.d	n.d	6.0	**9.6**	n.d	n.d
C24:1n-9	10.2	**33.1**	n.d	n.d	12.8	**21.3**	n.d	n.d
n-9 PUFA								
C20:3n-9	14.3	n.d	n.d	n.d	12.7	n.d	n.d	n.d
n-6 PUFA								
C18:2n-6	6.2	28.2	21.7	9.7	3.0	8.5	4.7	2.5
C18:3n-6	**10.2**	n.d	n.d	n.d	**11.1**	n.d	n.d	n.d
C20:3n-6	12.0	36.1	30.5	**14.0**	12.8	15.4	16.5	**12.3**
C20:4n-6	9.5	31.2	30.5	13.8	5.3	12.1	12.3	7.4
C22:5n-6	12.9	**30.2**	**36.9**	17.9	9.8	**15.9**	**24.3**	17.3
n-3 PUFA								
C18:3n-3	**13.5**	n.d	n.d	n.d	**13.2**	n.d	n.d	n.d
C20:5n-3	**11.5**	**34.1**	**35.0**	**31.3**	**7.0**	**19.6**	**24.1**	**23.1**
C22:5n-3	10.4	**33.9**	**35.8**	**15.8**	10.1	**19.2**	**19.2**	**18.2**
C22:6n-3	9.0	**32.9**	35.4	**12.9**	5.0	**13.5**	20.8	**6.7**

Bold data show the coefficient of variation of concentrations < 0.01 mg/g. n.d: concentrations < 0.001 mg/g not detected

Table 9.6: Inter-assay (n=23) of fatty acids during the study period expressed as coefficient of variation (CV in %)

	CV of the relative fatty aicd composition			
	PL	NEFA	TG	CE
Saturated fatty acids				
C14:0	6,9	**6,9**	**12,9**	**8,9**
C16:0	1,2	10,7	5,5	10,6
C17:0	5,2	**17,4**	n.d	n.d
C18:0	5,2	4,4	7,3	**19,0**
C20:0	10,7	**23,8**	n.d	n.d
C22:0	13,2	**30,5**	n.d	n.d
C24:0	6,4	**19,3**	n.d	n.d
Monounsaturated fatty acids				
C16:1n-7	4,0	**28,8**	**13,1**	**7,4**
C18:1n-9	3,2	7,6	10,0	5,4
C18:1n-7	9,1	**5,6**	**5,5**	**13,8**
C20:1n-9	9,2	**10,9**	n.d	n.d
C22:1n-9	11,3	n.d	n.d	n.d
C24:1n-9	14,8	**18,8**	n.d	n.d
Trans fatty acids				
C18:1 t	13,2	n.d	n.d	n.d
n-9 PUFA				
C20:3n-9	8,2	n.d	n.d	n.d
n-6 PUFA				
C18:2n-6	2,8	6,3	6,4	7,5
C18:3n-6	12,0	n.d	n.d	n.d
C20:2n-6	8,8	**8,3**	n.d	n.d
C20:3n-6	6,0	7,3	**14,8**	**13,1**
C20:4n-6	2,1	7,3	11,6	8,2
C22:2n-6	28,1	n.d	n.d	n.d
C22:5n-6	14,0	**9,1**	**19,1**	**17,4**
n-3 PUFA				
C18:3n-3	14,3	15,9	20,6	14,9
C18:4n-3	38,4	n.d	n.d	n.d
C20:3-n3	12,3	n.d	n.d	n.d
C20:5n-3	12,2	**29,8**	**39,8**	**25,8**
C22:5n-3	5,9	**10,3**	**18,6**	**29,5**
C22:6n-3	4,7	**8,7**	**16,5**	**16,4**

Bold data show the coefficient of variation of concentrations < 0.01 mg/g. n.d: concentrations < 0.001 mg/g not detected

Table 9.7: Fatty acid composition of phospholipids in term placenta of women in Germany, Spain and Hungary not receiving n-3 LC-PUFA or 5-MTHF supplement (% w/w, mean ± SEM). Identical superscripts indicate significant differences between populations. P < 0.05. (PUFA: polyunsaturated fatty acids).

	Germany	Spain	Hungary
N	15	26	13
Saturated fatty acids			
14:0	0,44 ± 0,02[a]	0,32 ± 0,01[a,c]	0,38 ± 0,02[c]
16:0	25,10 ± 0,16	25,35 ± 0,17	25,51 ± 0,18
17:0	0,33 ± 0,02	0,32 ± 0,01	0,29 ± 0,01
18:0	12,47 ± 0,25[a]	13,05 ± 0,18[a]	12,85 ± 0,13
20:0	0,30 ± 0,01	0,32 ± 0,02	0,29 ± 0,02
22:0	1,59 ± 0,04	1,62 ± 0,04	1,49 ± 0,06
24:0	0,75 ± 0,03	0,61 ± 0,02	0,60 ± 0,03
TOTAL	40,98 ± 0,22	41,59 ± 0,27	41,41 ± 0,26
Monounsaturated fatty acids			
16:1n-7	0,56 ± 0,04[a]	0,38 ± 0,02[a]	0,47 ± 0,03
18:1n-9	8,16 ± 0,13	8,27 ± 0,18	7,56 ± 0,19
18:1n-7	1,65 ± 0,07	1,55 ± 0,03[c]	1,74 ± 0,04[c]
20:1n-9	0,17 ± 0,01	0,18 ± 0,01	0,15 ± 0,01
22:1n-9	0,09 ± 0,00	0,10 ± 0,00	0,09 ± 0,01
24:1n-9	1,37 ± 0,04	1,51 ± 0,06	1,31 ± 0,07
TOTAL	12,01 ± 0,22	11,98 ± 0,22	11,32 ± 0,30
Trans fatty acids			
18:1 t	0,18 ± 0,02	0,14 ± 0,01	0,18 ± 0,01
n-9 PUFA			
20:3n-9	0,21 ± 0,01	0,18 ± 0,01	0,16 ± 0,01
n-6 PUFA			
18:2n-6	8,61 ± 0,33	9,25 ± 0,24	9,14 ± 0,38
18:3n-6	0,09 ± 0,00	0,09 ± 0,00	0,09 ± 0,00
20:2n-6	0,36 ± 0,01[a,b]	0,45 ± 0,01[a]	0,41 ± 0,02[b]
20:3n-6	5,52 ± 0,25	5,06 ± 0,15	5,29 ± 0,28
20:4n-6	22,10 ± 0,40	21,40 ± 0,25	22,90 ± 0,35
22:2n-6	0,05 ± 0,02	0,05 ± 0,00	0,05 ± 0,00
22:5n-6	1,96 ± 0,07	2,03 ± 0,06	1,70 ± 0,08
TOTAL n-6	38,68 ± 0,42	38,33 ± 0,25[c]	39,58 ± 0,38[c]
n-3 PUFA			
18:3n-3	0,03 ± 0,00	0,02 ± 0,00	0,02 ± 0,00
18:4n-3	0,01 ± 0,00[a]	0,04 ± 0,00[a,c]	0,01 ± 0,00[c]
20:3n-3	0,15 ± 0,01[b]	0,14 ± 0,00	0,12 ± 0,01[b]
20:5n-3	0,14 ± 0,01[b]	0,11 ± 0,01	0,06 ± 0,01[b]
22:5n-3	1,01 ± 0,05	0,98 ± 0,06	0,75 ± 0,03
22:6n-3	5,04 ± 0,28[b]	4,91 ± 0,19[c]	3,94 ± 0,18[b,c]
TOTAL n-3	6,38 ± 0,29[b]	6,20 ± 0,21[c]	4,91 ± 0,18[b,c]
n-6/n-3	6,1 ± 0,4[b]	6,2 ± 0,2[c]	8,1 ± 0,4[b,c]

Table 9.8: Comparison of the fatty acid profiles of placental PL between the supplementation groups without differentiation according to the country of origin (% w/w, mean ± SEM). Individual groups were supplemented with a placebo or n-3 LC-PUFA or 5-MTHF or n-3 LC-PUFA and 5-MTHF. Identical superscripts indicate significant differences between the supplemental groups. $P < 0.05$. Total saturated fatty acids (SFA) represent the sum of: C14:0, C16:0, C17:0, C18:0, C20:0, C22:0 and C24:0. Total monounsaturated fatty acids (MUFA): C16:1, C18:1, C20:1, C22:1, C22:0 and C24:1.

	Supplement			
	Placebo	n-3 LC-PUFA / 5-MTHF	n-3 LC-PUFA	5-MTHF
N	54	43	52	53
C18:1 t	0,15 ± 0,01	0,15 ± 0,01	0,15 ± 0,01	0,15 ± 0,01
C18:2n-6 (LA)	8,92 ± 0,21	9,20 ± 0,16	8,99 ± 0,16	9,08 ± 0,15
C18:3n-3 (ALA)	0,02 ± 0,00	0,02 ± 0,00	0,03 ± 0,00	0,02 ± 0,00
C18:3n-6	0,09 ± 0,00	0,08 ± 0,00	0,09 ± 0,00	0,09 ± 0,00
C18:4n-3	0,03 ± 0,00	0,02 ± 0,00	0,03 ± 0,00	0,02 ± 0,00
C20:2n-6	0,41 ± 0,01	0,42 ± 0,01	0,40 ± 0,01	0,42 ± 0,01
C20:3n-9	0,18 ± 0,01	0,16 ± 0,01	0,17 ± 0,01	0,17 ± 0,00
C20:3n-6 (DGLA)	5,24 ± 0,12	4,92 ± 0,11	4,95 ± 0,11	5,23 ± 0,15
C20:4n-6 (AA)	22,20 ± 0,27	21,57 ± 0,23	22,26 ± 0,26	22,14 ± 0,23
C20:3n-3	0,14 ± 0,00	0,14 ± 0,00	0,14 ± 0,00	0,14 ± 0,00
C20:5n-3 (EPA)	0,11 ± 0,01[a,b]	0,20 ± 0,02[a,c]	0,17 ± 0,01[b,d]	0,12 ± 0,01[c,d]
C22:2n-6	0,05 ± 0,00	0,04 ± 0,00	0,04 ± 0,00	0,05 ± 0,00
C22:5n-6 (DPA)	1,17 ± 0,08[a,b]	0,83 ± 0,08[a]	0,78 ± 0,05[b,d]	1,05 ± 0,05[d]
C22:5n-3 (DPA)	0,64 ± 0,02	0,69 ± 0,03	0,68 ± 0,02	0,71 ± 0,02
C22:6n-3 (DHA)	4,73 ± 0,14[a,b]	6,02 ± 0,16[a,c]	5,77 ± 0,15[b,d]	4,81 ± 0,13[c,d]
SUM SFA	41,86 ± 0,16	41,90 ± 0,22	41,44 ± 0,18	41,87 ± 0,21
SUM MUFA	11,74 ± 0,16	11,36 ± 0,15	11,41 ± 0,13	11,58 ± 0,14
SUM n-6	38,08 ± 0,20	37,06 ± 0,25	37,51 ± 0,23	38,06 ± 0,24
SUM n-3	5,66 ± 0,15[a,b]	7,10 ± 0,18[a,c]	6,81 ± 0,16[b,d]	5,76 ± 0,15[c,d]
n-6/n-3 ratio	6,7 ± 0,2[a,b]	5,2 ± 0,2[a,c]	5,5 ± 0,2[b,d]	6,6 ± 0,2[c,d]
18:2n-6/18:3n-3	420,5 ± 18,2	411,5 ± 18,3	389,0 ± 15,5	419,9 ± 20,1
20:4n-6/20:3n-6	4,4 ± 0,1	4,5 ± 0,1	4,6 ± 0,1	4,4 ± 0,1
22:6n-3/20:5n-3	57,5 ± 4,3	42,5 ± 4,3	45,4 ± 4,7	57,7 ± 5,1
20:4n-6/20:5n-3	295,7 ± 28,6[a]	162,9 ± 33,2[a,c]	194,1 ± 30,0	295,2 ± 19,2[c]
20:4n-6/22:6n-3	4,9 ± 0,2[a,b]	3,7 ± 0,1[a,c]	4,0 ± 0,1[b,d]	4,8 ± 0,2[c,d]

Table 9.9: Fatty acid profiles of placental lipid fractions in Germany, Spain and Hungary (% w/w, mean ± SEM). T-test was used to determine differences between placebo and n-3 LC-PUFA supplementation for each centre, respectively (*p<0.05). Total saturated fatty acids (SFA) represent the sum of: C14:0, C16:0, C17:0, C18:0, C20:0, C22:0 and C24:0. Total monounsaturated fatty acids (MUFA): C16:1, C18:1, C20:1, C22:1, C22:0 and C24:1. Total of n-6 poly unsaturated fatty acids (PUFA) was calculated with C18:2n-6; C18:3n-6, C20:3n-6, C20:4n-6, C20:5n-6 and 22:2n-6, total of n-3 PUFA with C18:3n-3, C18:4n-3, C20:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3.

	phospholipids					
	Germany		Spain		Hungary	
	placebo	n-3 fatty acids	placebo	n-3 fatty acids	placebo	n-3 fatty acids
N	24	24	54	46	29	25
C18:2n-6	8,27 ± 0,34	8,66 ± 0,19	9,28 ± 0,16	9,23 ± 0,17	9,07 ± 0,21	9,23 ± 0,23
C18:3n-6	0,09 ± 0,00	0,08 ± 0,00	0,09 ± 0,00	0,09 ± 0,00	0,09 ± 0,00	0,09 ± 0,00
C20:3n-6	5,46 ± 0,21	5,08 ± 0,14	5,12 ± 0,12	4,81 ± 0,12	5,27 ± 0,21	5,02 ± 0,13
C20:4n-6	22,12 ± 0,56	21,64 ± 0,34	21,73 ± 0,19	21,61 ± 0,21	23,03 ± 0,24	22,86 ± 0,42
C22:5n-6	1,15 ± 0,14	0,91 ± 0,12	0,94 ± 0,04	0,65 ± 0,03*	1,40 ± 0,05	0,98 ± 0,10*
C18:3n-3	0,03 ± 0,00	0,03 ± 0,00	0,02 ± 0,00	0,02 ± 0,00	0,02 ± 0,00	0,02 ± 0,00
C20:5n-3	0,16 ± 0,01	0,22 ± 0,02*	0,11 ± 0,01	0,19 ± 0,01*	0,07 ± 0,01	0,13 ± 0,02*
C22:5n-3	0,77 ± 0,03	0,81 ± 0,03	0,61 ± 0,01	0,66 ± 0,02	0,61 ± 0,02	0,62 ± 0,02
C22:6n-3	5,11 ± 0,21	6,16 ± 0,19*	4,94 ± 0,13	5,70 ± 0,17*	4,17 ± 0,16	5,96 ± 0,21*
total SFA	41,89 ± 0,43	41,57 ± 0,32	41,75 ± 0,17	41,87 ± 0,20	42,05 ± 0,15	41,03 ± 0,23
total MUFA	11,81 ± 0,21	11,56 ± 0,17	11,85 ± 0,15	11,63 ± 0,15	11,19 ± 0,19	10,77 ± 0,17
total n-6 PUFA	38,21 ± 0,46	37,77 ± 0,33	38,68 ± 0,17	38,11 ± 0,25	39,67 ± 0,21	39,27 ± 0,29
total n-3 PUFA	6,23 ± 0,22	7,37 ± 0,19*	5,86 ± 0,14	6,75 ± 0,18*	5,01 ± 0,17	6,88 ± 0,22*
n-6 / n-3	6,32 ± 0,25	5,21 ± 0,16*	6,80 ± 0,16	5,87 ± 0,20*	8,15 ± 0,25	5,88 ± 0,23*
PUFA / SFA	1,07 ± 0,02	1,09 ± 0,01	1,07 ± 0,01	1,08 ± 0,01	1,07 ± 0,02	1,12 ± 0,02
AA / DHA	4,48 ± 0,20	3,59 ± 0,13*	4,57 ± 0,13	3,98 ± 0,15*	5,72 ± 0,19	4,00 ± 0,20*
total (mg/g)	6,41 ± 0,25	7,33 ± 0,30	7,41 ± 0,14	7,21 ± 0,17	8,07 ± 0,16	7,96 ± 0,13

	non-esterified fatty acids					
	Germany		Spain		Hungary	
	placebo	n-3 fatty acids	placebo	n-3 fatty acids	placebo	n-3 fatty acids
N	24	24	54	46	29	25
C18:2n-6	7,93 ± 0,46	8,11 ± 0,38	9,93 ± 0,22	9,89 ± 0,21	8,80 ± 0,23	8,45 ± 0,28
C18:3n-6	0,11 ± 0,01	0,11 ± 0,02	0,14 ± 0,01	0,14 ± 0,01	0,10 ± 0,00	0,09 ± 0,01
C20:3n-6	4,99 ± 0,51	4,32 ± 0,20	4,99 ± 0,18	5,15 ± 0,19	5,01 ± 0,24	4,14 ± 0,20
C20:4n-6	16,89 ± 0,91	17,66 ± 0,88	18,97 ± 0,41	19,69 ± 0,39	19,55 ± 0,52	17,34 ± 0,61*
C22:5n-6	0,77 ± 0,07	0,69 ± 0,12	0,83 ± 0,06	0,64 ± 0,03*	1,32 ± 0,06	0,74 ± 0,06*
C18:3n-3	0,12 ± 0,01	0,13 ± 0,01	0,09 ± 0,00	0,09 ± 0,00	0,10 ± 0,01	0,10 ± 0,01
C20:5n-3	0,28 ± 0,03	0,59 ± 0,09*	0,35 ± 0,03	0,45 ± 0,03*	0,20 ± 0,02	0,33 ± 0,04*
C22:5n-3	0,66 ± 0,06	0,72 ± 0,05	0,48 ± 0,02	0,60 ± 0,02*	0,54 ± 0,04	0,53 ± 0,03
C22:6n-3	4,13 ± 0,28	4,93 ± 0,30*	4,27 ± 0,19	5,60 ± 0,22*	3,86 ± 0,25	5,02 ± 0,26*
total SFA	45,79 ± 1,41	45,04 ± 1,16	42,82 ± 0,86	40,84 ± 0,83	43,84 ± 0,80	44,82 ± 0,98
total MUFA	15,09 ± 1,06	14,82 ± 0,89	14,32 ± 0,33	14,22 ± 0,31	14,39 ± 0,77	15,84 ± 1,41
total n-6 PUFA	32,05 ± 1,54	32,10 ± 1,28	35,89 ± 0,61	36,64 ± 0,58	34,99 ± 0,62	31,67 ± 0,82*
total n-3 PUFA	5,39 ± 0,35	6,51 ± 0,39*	5,40 ± 0,21	6,83 ± 0,25*	4,82 ± 0,28	6,07 ± 0,31*
n-6 / n-3	6,29 ± 0,33	5,19 ± 0,26*	7,11 ± 0,26	5,73 ± 0,26*	7,73 ± 0,32	5,54 ± 0,29*
PUFA / SFA	0,87 ± 0,07	0,90 ± 0,06	1,01 ± 0,04	1,11 ± 0,04	0,93 ± 0,03	0,85 ± 0,03
AA / DHA	4,34 ± 0,25	3,79 ± 0,23	4,82 ± 0,19	3,78 ± 0,17*	5,45 ± 0,26	3,69 ± 0,23*
total (mg/g)	0,30 ± 0,03	0,31 ± 0,02	0,25 ± 0,01	0,28 ± 0,01	0,41 ± 0,02	0,37 ± 0,02

Table 8.9 (contd.):

	triglycerides					
	Germany		Spain		Hungary	
	placebo	n-3 fatty acids	placebo	n-3 fatty acids	placebo	n-3 fatty acids
N	24	24	54	46	29	25
C18:2n-6	9,40 ± 0,42	9,21 ± 0,52	10,85 ± 0,25	11,50 ± 0,25	12,03 ± 0,30	11,41 ± 0,36
C18:3n-6	0,20 ± 0,01	0,18 ± 0,02	0,24 ± 0,01	0,27 ± 0,01	0,21 ± 0,01	0,18 ± 0,01
C20:3n-6	6,28 ± 0,52	5,97 ± 0,32	5,98 ± 0,25	5,99 ± 0,28	8,10 ± 0,53	7,12 ± 0,32
C20:4n-6	12,91 ± 0,57	13,25 ± 0,45	11,94 ± 0,31	12,24 ± 0,33	15,72 ± 0,50	14,86 ± 0,53
C22:5n-6	1,06 ± 0,09	0,76 ± 0,06*	0,96 ± 0,06	0,74 ± 0,04*	1,69 ± 0,12	1,18 ± 0,12*
C18:3n-3	0,19 ± 0,01	0,21 ± 0,02	0,14 ± 0,01	0,17 ± 0,01	0,17 ± 0,01	0,16 ± 0,01
C20:5n-3	0,24 ± 0,02	0,37 ± 0,03*	0,23 ± 0,03	0,40 ± 0,05*	0,15 ± 0,01	0,25 ± 0,03*
C22:5n-3	0,86 ± 0,09	1,12 ± 0,13	0,63 ± 0,05	0,86 ± 0,06*	0,71 ± 0,05	0,85 ± 0,06
C22:6n-3	4,92 ± 0,49	7,10 ± 0,79*	4,60 ± 0,32	6,63 ± 0,48*	4,49 ± 0,36	6,81 ± 0,52*
total SFA	40,21 ± 0,88	40,07 ± 1,12	39,82 ± 0,92	39,62 ± 0,71	37,21 ± 0,49	37,94 ± 0,70
total MUFA	21,67 ± 1,05	19,32 ± 0,55	18,86 ± 0,47	19,35 ± 0,40	17,64 ± 0,73	16,91 ± 0,56
total n-6 PUFA	29,88 ± 1,13	29,78 ± 0,78	30,18 ± 0,52	31,12 ± 0,48	37,10 ± 0,76	34,75 ± 0,77
total n-3 PUFA	6,32 ± 0,59	8,93 ± 0,92*	5,66 ± 0,37	8,13 ± 0,55*	5,61 ± 0,41	8,15 ± 0,58*
n-6 / n-3	5,58 ± 0,44	3,99 ± 0,32*	6,32 ± 0,33	4,64 ± 0,32*	7,49 ± 0,49	4,74 ± 0,32*
PUFA / SFA	0,93 ± 0,05	1,01 ± 0,06	0,94 ± 0,03	1,02 ± 0,04	1,16 ± 0,04	1,15 ± 0,04
AA / DHA	3,29 ± 0,34	2,37 ± 0,25	3,13 ± 0,18	2,30 ± 0,17*	4,12 ± 0,34	2,49 ± 0,21*
total (mg/g)	0,17 ± 0,01	0,15 ± 0,01	0,15 ± 0,01	0,19 ± 0,01	0,19 ± 0,01	0,19 ± 0,01

	cholesterolesters					
	Germany		Spain		Hungary	
	placebo	n-3 fatty acids	placebo	n-3 fatty acids	placebo	n-3 fatty acids
N	24	24	54	46	29	25
C18:2n-6	29,64 ± 1,11	30,04 ± 1,05	31,05 ± 0,63	32,47 ± 0,83	31,35 ± 0,97	31,00 ± 1,10
C18:3n-6	0,79 ± 0,09	0,74 ± 0,09	1,06 ± 0,06	0,84 ± 0,05	0,61 ± 0,03	0,59 ± 0,03
C20:3n-6	3,57 ± 0,22	3,31 ± 0,22	3,62 ± 0,15	3,42 ± 0,18	4,27 ± 0,35	3,93 ± 0,29
C20:4n-6	10,16 ± 0,48	9,83 ± 0,51	9,86 ± 0,22	9,31 ± 0,26	11,50 ± 0,29	10,76 ± 0,36
C22:5n-6	0,85 ± 0,09	0,64 ± 0,05*	0,95 ± 0,06	0,68 ± 0,04*	1,34 ± 0,10	1,06 ± 0,09*
C18:3n-3	0,44 ± 0,03	0,40 ± 0,03	0,29 ± 0,01	0,34 ± 0,02	0,32 ± 0,02	0,30 ± 0,03
C20:5n-3	0,33 ± 0,04	0,52 ± 0,05*	0,28 ± 0,03	0,50 ± 0,03*	0,13 ± 0,01	0,30 ± 0,04*
C22:5n-3	0,30 ± 0,03	0,39 ± 0,05	0,21 ± 0,02	0,30 ± 0,02*	0,28 ± 0,03	0,29 ± 0,03
C22:6n-3	2,64 ± 0,16	3,74 ± 0,26*	2,92 ± 0,15	3,91 ± 0,20*	3,45 ± 0,20	4,27 ± 0,32*
total SFA	24,45 ± 0,56	24,10 ± 0,47	23,30 ± 0,37	22,56 ± 0,46	23,43 ± 0,60	24,26 ± 0,74
total MUFA	25,92 ± 0,53	25,38 ± 0,47	25,56 ± 0,38	24,63 ± 0,46	22,63 ± 0,41	22,26 ± 0,61
total n-6 PUFA	44,89 ± 0,88	44,59 ± 0,88	46,39 ± 0,50	46,81 ± 0,67	48,41 ± 0,72	46,99 ± 0,88
total n-3 PUFA	3,70 ± 0,20	5,05 ± 0,33*	3,71 ± 0,16	5,06 ± 0,22*	4,18 ± 0,22	5,17 ± 0,35*
n-6 / n-3	12,88 ± 0,67	9,96 ± 0,90*	13,73 ± 0,60	10,16 ± 0,52*	12,46 ± 0,70	10,49 ± 0,95*
PUFA / SFA	2,03 ± 0,07	2,09 ± 0,07	2,19 ± 0,05	2,36 ± 0,07	2,31 ± 0,09	2,22 ± 0,09
AA / DHA	4,20 ± 0,33	2,98 ± 0,28*	3,88 ± 0,24	2,65 ± 0,15*	3,58 ± 0,20	2,95 ± 0,30
total (mg/g)	0,35 ± 0,04	0,31 ± 0,03	0,40 ± 0,03	0,41 ± 0,03	0,52 ± 0,04	0,46 ± 0,03

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11 Publication and abstract list

Publications:

Klingler M, Demmelmair H, Larque E, and Koletzko B. (2003) Analysis of FA contents in individual lipid fractions from human placental tissue. *Lipids 38*, 561-566.

Klingler M, Demmelmair H, Campoy C, Jiménez M, Decsi T, Veszprémi B, and Koletzko B. Effect of fish oil and folate supplementation during pregnancy on fatty acid patterns in placental phospholipids in three European countries (submitted EJCN).

Klingler M, Demmelmair H, Campoy C, Jiménez M, Decsi T, Veszprémi B, and Koletzko B. Influence of habitual diet and additional docosahexaenoic acid intake during pregnancy on the fatty acid profile of placental lipid fractions (in preparation).

Klingler M, Blaschitz A, Campoy C, Jiménez M, Molloy AM, Scott JM, Dohr G, Demmelmair H, Koletzko B, and Desoye G. The effect of docosahexaenoic acid and folic acid supplementation on the placental apoptosis and proliferation (in preparation).

Larque E, Demmelmair H, Klingler M, De Jonge S, Bondy B, and Koletzko B. Expression pattern of FATP-1, FATP-4 and H-FABP genes in human term placenta (submitted).

Hartl D, Klingler M, Demmelmair H, Campoy C, Diaz JM, Hector A, Prell C, Reinhardt D, Schendel DJ, Krauss-Etschmann S, Koletzko B. Association of T cell cytokine and chemokine receptor mRNA expressions in cord and maternal blood with placental n-3 LC-PUFA content (in preparation).

Abstracts

Klingler M, Demmelmair H, Campoy C, Desci T, Koletzko B. Low eicosapentaenoic acid concentrations in fish oil supplements do not influence the arachidonic acid contents in placental lipids. WCPGHAN 2004 (accepted).

Hartl D, Klingler M, Demmelmair H, Campoy C, Diaz JM, Hector A, Prell C, Reinhardt D, Schendel DJ, Krauss-Etschmann S, Koletzko B. Association of T cell cytokine and chemokine receptor mRNA expressions in cord and maternal blood with placental n-3 LC-PUFA content. WCPGHAN 2004 (accepted).

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Curriculum Vitae

06.04.1970	born in Illertissen,
09/1976 – 07/1980	Primary School Neu-Ulm
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09/1987 – 07/1990	Professional training as a chef; Journeyman's certificate at the Restaurant "Florianstuben", Ulm, Germany
09/1990 – 06/1991	Chef in Colombi Hotel, Freiburg
07/1991 – 06/1992	Military Service
08/1992 – 07/1993	Graduation from Highschool (A-Levels), Merian-Gymnasium Freiburg, Germany
10/1993 – 09/1995	Pre-diploma: Technical School Fulda, Germany; basics in natural sciences and economics
10/1995 – 05/2001	Diploma: Justus-Liebig-University Gießen, Germany; principal subjects: nutrition, biochemistry, economics, food science; Diploma Thesis at Nestlé R&D Centre, Weiding, Germany, Nestlé Research Centre, Lausanne, Switzerland and Justus-Liebig-University Gießen, Germany: *Degradation of fructans in baby food*
12/2001 – 02/2004	Dissertation at Dr. von Hauner Children's Hospital, Munich, Germany and Karl Franz University Graz, Austria: *The influence of an additional intake of docosahexaenoic acid and folate during second half of pregnancy on placental fatty acid patterns, apoptosis and proliferation*
02/2004 – 06/2004	Paediatric Immunology, Dr. von Hauner Children's Hospital, Munich, Germany
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